Chapter 8

Applications of Moving Boundary Electrophoresis to Protein Systems

I. Introduction

The method of Tiselius electrophoresis as applied to protein systems can yield a variety of information. The quantities measured experimentally are the areas under the boundaries formed during migration, the displacements of the boundaries, the shapes of the boundaries and their degree of spreading. From these measurements, it should be possible to obtain information on the zeta potential and charge of a protein molecule, the degree of homogeneity of a protein, the composition of a mixture of proteins, the effect of various reagents and treatments on the protein and the nature and

degree of interaction of the proteins with themselves, with other proteins, and with ions or small molecules present in the solution. In previous chapters, a thorough theoretical treatment of these various topics has been presented. It is the purpose of this chapter to show by examples how some of these theories may be applied to actual systems and how an electrophoretic pattern may be properly interpreted. First we will treat the relation of mobility to the charge in the protein molecule and to its surrounding medium. Then, various types of heterogeneity will be treated, and finally the problems of interaction and modification of the molecule will be presented.

II. Effect of Buffers on Electrophoresis

A. Relation between Charge and Mobility

In general, there has been little activity in the use of Tiselius electrophoresis for the determination of zeta potentials and electric charges carried by proteins. One of the reasons for this apparent lack of interest is that the interpretation of electrophoretic mobilities in terms of charge is somewhat equivocal. There are some difficulties in the theoretical treatment, as shown by Overbeek in Chapter 1 of this monograph. Two excellent reviews of the theory of electrophoretic migration of colloids are available; one is in the book by Abramson *et al.* (1); the other is in a recent chapter by Overbeek (2). Only the results of the theory will be presented here, the reader being referred to Chapter 1 in this volume and the two reviews for a detailed treatment.

The relation between mobility, u, in cm.^{2}/volt-sec., and the zeta potential, ζ, as developed by Smoluchowski (3), Debye and Hückel (4), Hückel (5), and Henry (6) for a spherical particle of radius a is

$$u = \frac{D\zeta}{6\pi\eta} f(\kappa a)$$

$$f(\kappa a) = 1 + \frac{\kappa a^2}{16} - \frac{5\kappa^3 a^2}{16} \quad \frac{5\kappa^3 a^3}{48} - \frac{\kappa^4 a^4}{96} + \frac{\kappa^5 a^5}{96} - \left(\frac{\kappa^4 a^4}{8} - \frac{\kappa^6 a^6}{96}\right) e^{\kappa a} \int_{\infty}^{\kappa a} \frac{e^{-t}}{t}\, dt \quad (1)$$

where D is the dielectric constant, η is the viscosity of the liquid, $1/\kappa$ is the thickness of the Debye-Hückel ion atmosphere

$$1/\kappa = \left(\frac{DkT}{4\pi e^2 \Sigma u_i z_i^2}\right)^{1/2} \quad (2)$$

k is Boltzmann's constant, T the absolute temperature, e the elementary

charge, z_i the valence of the ions of species i, and n_i their number per cubic centimeter.

The function $f(\kappa a)$ approaches $\frac{3}{2}$ for spheres which are large when compared to the thickness of the double layer and 1 for very small spheres. Values of this function have been tabulated by Henry (6) [see also (1)] as a function of the ionic strength.

Henry's equation neglects the distortion of the double layer by relaxation effects. Overbeek (2) has shown that the correction for this effect is largest for intermediate values of κa, in the region of greatest interest for the protein chemist, i.e. for particles of dimensions between 10Å and 1μ and values of κ between 10^5 and $10^{7.5}$. A detailed treatment of this effect is available in Overbeek's articles.

The theory of ionic atmospheres (7) may be used to express the zeta potential in terms of the total charge, Q, on the protein. Taking into account the finite radius, a_i, (8) of the ions in the supporting electrolyte, one obtains

$$Q = D\zeta a\left[\frac{1 + \kappa(a + a_i)}{1 + \kappa a_i}\right] \tag{3}$$

For point charges this reduces to

$$Q = D\zeta a(1 + \kappa a) \tag{4}$$

Combination of equation (1) with equation (3) or (4) gives the relation between the charge on the protein and its electrophoretic mobility. In the application of the theory to actual protein systems several difficulties are encountered. The principal one is that the theory has not been extended to particles which are ellipsoidal in shape. Gorin [cf. reference (1)] has determined the relation between charge and zeta potential for cylindrical particles. This equation, which has been applied to ellipsoidal particles, is

$$u = \frac{Qf(\kappa a)(1 + \kappa a_i)}{6\pi\eta a[1 + \kappa(a + a_i)f(\kappa,a/b)C]} \tag{5}$$

The function $f(\kappa,a/b)$ has been tabulated by Gorin (1). It takes into account the axial ratio, a/b. C is a dimensional constant.

Little experimental work has been done on the relation of electrophoretic mobility to protein charge. Longsworth has determined the mobilities of ovalbumin over the pH range of 1.8–12.8 at 0.1 ionic strength (9) and pH 3.1–11.7 at 0.01 ionic strength (10). A comparison of his data at 0.1 ionic strength with titration data of Cannan *et al.* (11) is given in Fig. 1. In this figure, the number of equivalents of acid bound per gram of protein are plotted as ordinates on the left-hand side, the scale being shifted vertically so that the point of zero binding coincides with the electrophoretic isoelectric point of ovalbumin at 0.1 ionic strength, i.e.

pH 4.58. The mobilities are indicated by the circles, using the right-hand ordinate scale. An excellent parallelism between the two sets of data can be seen. When the charge was calculated from the mobility data, using equations (1) and (4), it was found that the electrophoretic charge values

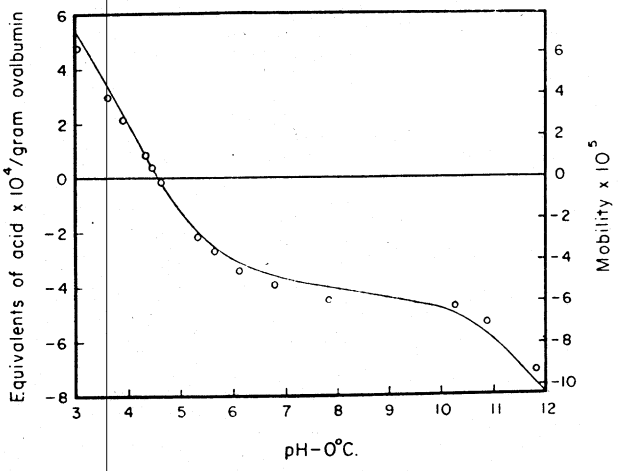


FIG. 1. pH-Mobility and titration curves of ovalbumin in 0.1 ionic strength (9). The mobilities are indicated by the circles, the curve represents the properly normalized titration curve.

were only 60% of the titration charge over the entire pH range. Correction for the relaxation effects amounts to only 5% (2), while assumption of a cylindrical shape for the protein molecule increases the charge by 20% (1). Thus, a significant lack of quantitative agreement still exists.

A comparison has been made by Cannan and associates (12) of their titration data on β-lactoglobulin with electrophoretic data of Pedersen (13) at 0.2 ionic strength in the pH range between 3.2 and 8.9. In this case, the electrophoretic charge was found to be 85% of the titration charge. The better agreement in this case is explained by these authors as being due to the absence in β-lactoglobulin of the discrepancy between isoelectric and isoionic points found in ovalbumin.

A more recent study of this nature was carried out on trypsin by Duke and co-workers (14). Their results are presented in Fig. 2. For the sake of convenience, these authors chose pH 6.3 as the common point of reference for the two curves, so that the acid-binding capacity of the isoelectric protein is given by the sum of all the hydrogen and hydroxyl ions bound below pH 10.8, the isoelectric point. Again there is an excellent parallelism between the electrophoretic mobility and the acid-binding capacity of this protein. No comparison was made, however, of the charges calculated from

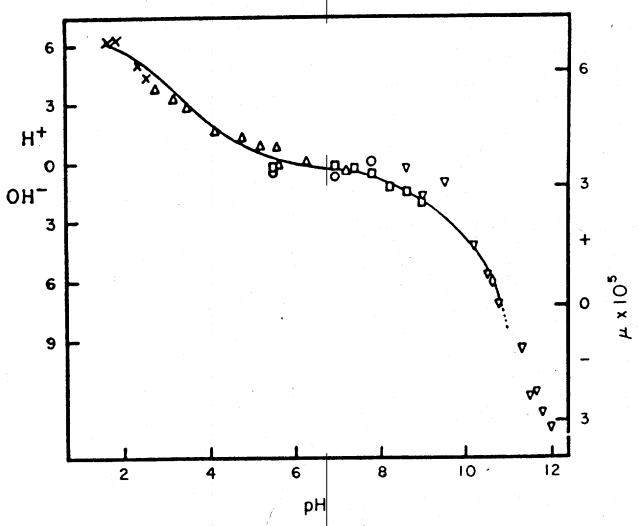


FIG. 2. Comparison of electrophoretic mobilities and dissociation curves of trypsin. Solid line: dissociation curve; points: electrophoretic mobilities in various buffers, ionic strength 0.13 (14). X, NaCl-HCl; △, NaAc-HCl; □, $CaCl_2$-barbiturate; ○, $MgCl_2$-barbiturate; ▽, $CaCl_2$-glycine; ◇, $CaCl_2$-NH_4Cl.

the two sets of data. The discrepancy between mobility and acid-binding capacity of some points measured in the presence of calcium ions may be attributed to the binding of these ions by the enzyme (14).

Velick (15) has used equation (5) to calculate the net charge of aldolase from the observed mobility in phosphate and acetate buffers of various ionic strengths. From these data he evaluated the number of phosphate ions bound per protein molecule and obtained good agreement with values calculated from dialysis equilibrium experiments. Comparison with titration data, however, resulted in a discrepancy that required eight times as many bound charges as found in dialysis equilibrium in order to reconcile the two sets of data. This discrepancy has been attributed by Velick to the probable effects of the nonavailability of some groups and to details of the molecular configuration.

The difference between titration and electrophoretic charge data has not been accounted for quantitatively. It would appear that the binding of counterions increases as the protein acquires a net charge. The zeta potential obtained in electrophoresis refers to the surface of shear. At high ionic strength, part of the counterions are present within this surface and by a steric effect diminish the electrophoretic charge, while they are not detected by other methods, such as titration experiments.

In recent years, the theory of titration curves has been developed considerably. A detailed treatment is given by Linderstrøm-Lang and

Nielsen in Chapter 2. The relation (16, 17) normally used to relate pH to the dissociation properties of ionizable groups on proteins is

$$\text{pH} - \log \frac{r_i}{n_i - r_i} = (\text{p}K_{\text{int.}})_i - 0.868wZ \tag{6}$$

where n_i is the number of ionizable groups of type i, r_i is the number of groups of type i dissociated at any pH, $(\text{p}K_{\text{int}})_i$ is the intrinsic dissociation constant of group i, and w takes into account the electrostatic interaction between the net charge Z of the protein at any pH and the dissociating hydrogen ions, and is equal to

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \tag{7}$$

where e is the protonic charge, D is the dielectric constant of the solvent, b is the radius of the spherical protein molecule, and a is the distance to which salt ions are excluded.

In the analysis of titration curves, the binding of ions other than hydrogen and hydroxyl is not taken into account. Furthermore, it is generally assumed that all ionizable groups are distributed evenly over the surface of the spherical molecule. Tanford (18, 19) has shown, recently, that the dissociation properties of ionizable groups on a protein molecule are quite sensitive to the location of the dissociable site with respect to the surface of the molecule, to the distribution of the ionizable sites with respect to one another, and to the ionic strength of the medium. Any change in these variables either in titration or electrophoretic experiments could result in significant variations of the net charge on the protein molecule.

Thus, while some data are available to show at least a semiquantitative relationship between charge and mobility, more work is necessary; especially desirable would be a series of studies correlating titration data with electrophoretic mobilities at different ionic strength and with ion binding taken into account.

B. Effect of Ionic Strength on Mobility

The Henry equation [equation (1)] has only a small explicit dependence on the ionic strength of the supporting electrolyte. $f(\kappa a)$ approaches $\frac{3}{2}$ at high salt concentrations and 1 at low concentrations. If one assumes, however, that the total charge on the protein is unaffected by the ionic strength, the zeta potential, and consequently the electrophoretic mobility, becomes strongly dependent on the ionic strength:

$$u = \frac{Kf(\kappa a)}{(\Gamma/2)^{1/2}} \tag{8}$$

where $\Gamma/2 = \frac{1}{2}\Sigma n_i Z_i^2$ is the ionic strength, and K is a constant.

Tiselius and Svensson (20) have measured the mobility of ovalbumin as a function of ionic strength at pH 7.10 and compared it with values calculated using the Henry equation. Their results indicate excellent agreement between the experimental and calculated values.

The effect of ionic strength on the isoelectric point has been studied for a number of proteins. Results presented in Fig. 3 for ovalbumin (20),

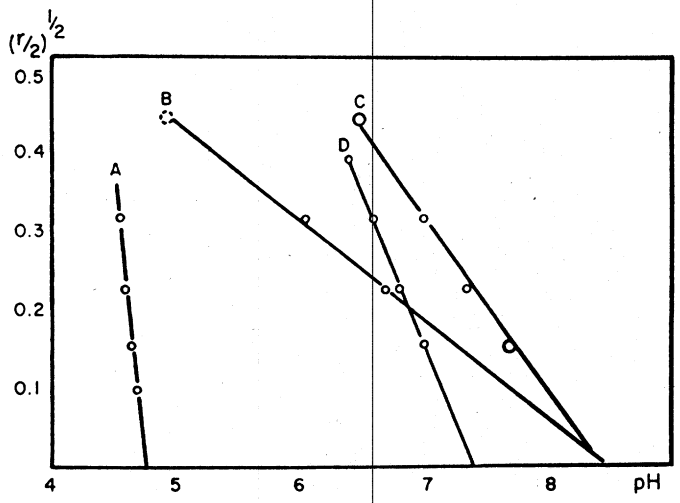


FIG. 3. Electrophoretic isoelectric points as a function of the square root of ionic strength. Curve *A*, ovalbumin in acetate; curve *B*, aldolase in phosphate; curve *C*, aldolase in acetate; curve *D*, horse carboxyhemoglobin in phosphate (15).

carboxyhemoglobin (21), and aldolase (15) show a straight-line dependence of the isoelectric point on the square root of ionic strength. A similar linear relationship has been observed by Adair and Adair (22) in measurements of membrane potentials in the case of sheep hemoglobin. Such a dependence has been attributed by Tiselius and Svensson (20), at least partially, to the combination of buffer ions with the protein. Longsworth and Jacobsen (23) have shown that the data of Tiselius and Svensson (20) on ovalbumin may be represented, with an average deviation of 0.002 pH unit, by the relation

$$\mathrm{p}I = \mathrm{p}I_0 - \frac{a(\Gamma/2)}{1 + b(\Gamma/2)} \qquad (9)$$

with $\mathrm{p}I_0 = 4.752$, $a = 5.0$, $b = 20$. This relation was obtained by combining a Langmuir adsorption isotherm with the assumption that the displacement of the isoelectric pH with increasing ionic strength is proportional to the change in net charge that accompanies the binding of buffer ions by the protein, i.e., $\mathrm{p}I = \mathrm{p}I_0 - k\Delta Z$.

A strong dependence of isoelectric point on ionic strength of human γ_1- and γ_2-globulins has been observed by Alberty (24), while similar data on bovine serum albumin and β-lactoglobulin has been obtained by Longsworth and Jacobsen (23). These authors point out the importance of the effect of bound salt ions on the ionization constants of ionizable groups on the protein. Anion binding is accompanied, at constant pH, by the binding of additional protons. Thus, if on partial substitution of the salt NaS for salt NaR at constant pH and ionic strength, s anions of S species are bound by each molecule of protein, displacing Δr of the R anions that are bound in solution of pure NaR and inducing the addition of Δh protons, the change in net charge is:

$$\Delta Z = -s + \Delta r + \Delta h \tag{10}$$

In a case where the amino acid composition is such that the protein is relatively weakly buffered in the isoelectric region, changes in bound charges are accompanied by a large pH shift, which is required for electrical compensation. In the case of aldolase, Velick (15) has found that the isoelectric point determined in 0.2 ionic strength phosphate buffer is 4 pH units less than the value calculated from amino acid composition.

C. The Moving Boundary Equation

In the electrophoretic analysis of protein mixtures for composition, it is found often that the apparent relative concentrations of components vary with the ionic strength of the buffer and the total protein concentration. These observations are the result of anomalies encountered in moving boundary electrophoresis. A thorough discussion of the theory of moving boundaries (25, 26) is given by Longsworth in Chapter 3 of this book. Using the notation of Longsworth the distribution of component j across boundary $\alpha\beta$ is given by

$$\frac{c_j^{\alpha}}{c_j^{\beta}} = \left(\frac{r_j}{\sigma^{\beta}} - V^{\alpha\beta}\right) \Big/ \left(\frac{r_j}{\sigma^{\alpha}} - V^{\alpha\beta}\right) \tag{11}$$

where r_j is the relative mobility, σ is the relative conductivity, $V^{\alpha\beta}$ is the volume swept out by boundary $\alpha\beta$ on the passage of one Faraday equivalent of electricity. The superscripts refer to the phase. Designating as component 1 the one which disappears across boundary $\alpha\beta$, it is found for component 2 that

$$\frac{c_2^{\alpha}}{c_2^{\beta}} = \left(\frac{r_2}{\sigma^{\beta}} - \frac{r_1}{\sigma^{\beta}}\right) \Big/ \left(\frac{r_2}{\sigma^{\alpha}} - \frac{r_1}{\sigma^{\beta}}\right) \tag{12a}$$

$$\frac{c_2^{\alpha}}{c_2^{\beta}} = 1 - \left[\frac{r_2}{r_2 - r_1} \cdot \frac{\sigma^{\beta} - \sigma^{\alpha}}{\sigma^{\alpha}}\right] + \cdots \tag{12b}$$

The consequences of these equations are discussed in detail by Longsworth (see Chapter 3). Several generalizations, however, can be made on the basis of the above equations. There is a concentration change across the boundary of any component with an electrophoretic mobility, and electrophoresis does not give in general the correct analysis of a mixture. Equation (12b) shows that the measured areas will deviate most seriously from the correct composition for two components with similar mobilities. This has been shown for hemoglobin, (27), β-lactoglobulin (28), and ovalbumin (29), among others. In order to analyze such systems, one may have to use calibration graphs of area distribution against known composition (27, 28, 30) or extrapolation of observed values to zero protein concentration at constant ionic strength (31) or to zero values of the ratio of protein concentration to ionic strength (32, 33).

Cann (29) has shown that in the case of ovalbumin the Dole theory (25) accounts adequately for the apparent composition of the protein as a function of concentration. (A detailed treatment of the heterogeneity of ovalbumin is given in Section III, A.) In this work the "true" electrophoretic distribution of components was determined by extrapolation of apparent distributions at finite concentrations to zero protein concentration at constant ionic strength. The deviations of the apparent from the extrapolated relative concentrations of component A_1 of ovalbumin when plotted against protein concentration were found to fall on straight lines, passing through the origin, as shown in Fig. 4. Theoretical deviations for two ovalbumin samples were calculated using the equations of Dole and are compared with experimental data in Fig. 4. The author concludes

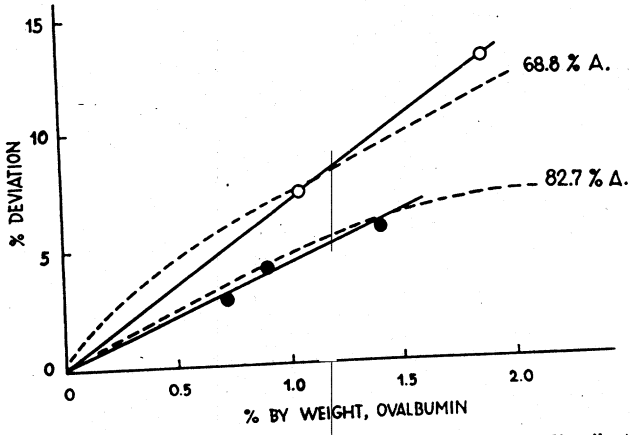


FIG. 4. Deviation of apparent from "true" electrophoretic distributions in ovalbumin (29); solid line, experimental deviations; broken curve, deviations calculated from the equation of Dole.

that the degree of agreement obtained is an adequate check of the Dole theory as applied to ordinary electrophoretic analyses of proteins.

Longsworth (31) has applied the theory of moving boundaries to systems consisting of a single protein, as well as to protein mixtures, and has shown that the net charge of ovalbumin may be calculated from the change in buffer concentration across the ϵ-boundary. A detailed analysis of this treatment is presented in Chapter 3 of this volume. Hoch (34) has examined the problem of boundary anomalies and concluded that relative concentrations in protein mixtures as deduced from apparent concentration changes at the boundaries are grossly erroneous when the difference in component mobilities is small. He further concluded that examination of a protein for homogeneity should be carried out at low concentrations, where the separation of boundaries is greatest. This can be carried out best with interference fringe optics, a procedure described in Chapter 4.

The foregoing analyses of moving boundary systems indicate that measured mobilities and areas should be extrapolated to zero protein concentrations. At zero concentration there will be no conductivity changes across the boundaries and the measured mobilities for all components will be correct. From equation (11) it can be seen that the ratio of concentrations across the boundary will become unity for all components which do not disappear across the boundary when the conductivities in the two phases are equal. This implies that measured areas which have been extrapolated to zero protein concentration will be correct.

One should add a word of caution, however. Equation (11) is based on the assumption that the relative mobilities are independent of phase. This appears to be a good assumption for strong electrolytes. It may be a rather poor one for weak electrolytes such as proteins. It has not been established yet that the analysis obtained by extrapolation to zero concentration is the correct one when dealing with weak electrolytes.

It has been shown that there are conductivity changes across the boundaries in electrophoresis. Since proteins in general bind hydrogen ions, there will be also pH gradients across the boundaries. The net result of these changes across the boundaries is either an increase or a decrease in the migration velocity of the protein across the boundaries and a consequent sharpening or broadening of the boundaries. These effects are treated in detail in Chapter 3.

Ideally the patterns in the two limbs should be mirror images. In practice the pH and conductivity changes across the boundaries cause deviations from enantiography. This phenomenon is common enough, so that the tendency is to overlook it. It should be realized, however, that marked deviations from enantiography are to be avoided since they are

indicative of errors in the analysis. When the deviations are very marked it should be verified that the two limbs do approach mirror images at zero protein concentration.

The resolving power of the Tiselius method can be increased by taking advantage of the larger mobility differences and higher field strengths attainable at low ionic strength. Due to the presence of boundary-sharpening effects in the rising limb, best resolution into components is often obtained on that side. This can be used to advantage in the qualitative detection of components in a system.

Unfortunately, since the electrophoretic anomalies also increase at low ionic strength, in striving for increased resolution one is forced to compromise. Using 0.5% protein solutions, the results become equivocal below 0.05 ionic strength. The highly precise interference optics which are available now make it possible to work with lower protein concentrations. Using protein concentrations of the order of 0.1%, one may be able to take advantage of the improved resolution available at 0.01 ionic strength. Careful studies should be made, however, to see if the artifacts arising because of unstable boundaries and electroosmosis can be kept within tolerable limits under these conditions.

III. Protein Heterogeneity

The most important use of electrophoresis is the analysis of heterogeneous mixtures. It is particularly useful in following fractionations of mixtures. A detailed discussion of the methods of obtaining relative concentrations of proteins from electrophoretic patterns is presented in Chapter 3. Cautions to be exercised, owing to the presence of boundary anomalies, have been described above and are treated in detail in Chapter 3. The application of electrophoresis to the analysis of complex mixtures of proteins and for following the progress of protein fractionations is discussed in Chapters 3, 4, and 7 of this book. Tiselius electrophoresis, however, is also a very powerful criterion of the homogeneity of single "pure" proteins. In recent years, with the use of this method, a number of proteins have been critically examined for homogeneity with the results showing a greater or smaller degree of heterogeneity in all. A few typical examples taken from these studies are discussed below. In most of the systems to be described below the differences between the discrete proteins are small and the resulting electrophoretic heterogeneity might not be immediately obvious. The electrophoretic resolution is often poor and might require a set of special conditions for detection. In some cases, resolution into components never occurs and the heterogeneity can be detected only from the spreading of the boundary.

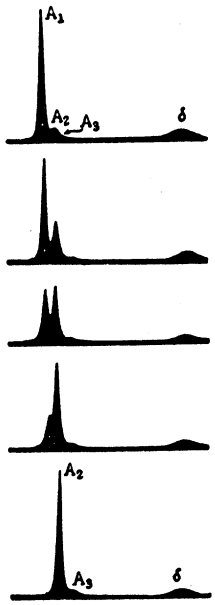
One of the most complete electrophoretic investigations of the homogeneity of a protein has been carried out on ovalbumin. As a result, a thorough understanding of that system is available now. The electrophoretic heterogeneity of ovalbumin has been investigated by a number of workers (29, 35–39). Longsworth (35) first pointed out that crystalline ovalbumin resolved into two electrophoretic components. A similar observation was made by Tiselius and Eriksson-Quensel (36). A detailed study of this problem (37, 38) revealed the presence of three components, designated as A_1, A_2, and A_3 in decreasing order of mobility on the alkaline side of the isoelectric point. These were found to vary in relative concentration between preparations. MacPherson and associates (38) further demonstrated that prolonged storage resulted in the disappearance of component A_1, accompanied by a large increase in the amount of A_2 present in the system. Perlmann (40–42) carried out a detailed study of the difference in components of ovalbumin and of the mechanism of their interconversion. She found that A_1, A_2, and A_3 differ primarily in their phosphorus content and have 2, 1, and 0 phosphate residues per mole, respectively. The mobilities of the three components at pH 6.8 in phosphate buffer of 0.1 ionic strength and their isoelectric points are given in Table I. Such differences in mobility and isoelectric pH have been shown to correspond to a change in the net charge of -2 per phosphorus atom (42). Dephosphorylation of A_1 to A_2 was carried out with prostate phosphatase at pH 5.35 or intestinal phosphatase at pH 9, with the accompanying electrophoretic changes shown in Fig. 5. Conversion of A_2 to A_3 could be carried out only with intestinal phosphatase at pH 5.3. This difference in splitting may

TABLE I

ELECTROPHORETIC PROPERTIES OF OVALBUMINS AND PLAKALBUMINS[a]

Protein	A_1	A_2	A_3	P_1	P_2	P_3
Crystal form	Needles	Needles	Needles	Plates	Plates	Plates
Atoms phosphorus per mole of protein	2	1	0	2	1	0
$u \times 10^5$ cm.^{2}/ sec. v.[b]	−6.1	−5.2	−4.5	−5.5	−4.6	−3.8
Isoelectric point at 0.1 ionic strength	4.58	4.65	4.74	4.72	4.8	

[a] According to Perlmann (42).
[b] Electrophoresis in pH 6.8 phosphate buffer of 0.1 ionic strength.



Time in hours	Electrophoretic composition	Atoms phosphorus per mole protein	
		Computed	Observed
0	85% A_1 14% A_2 Trace A_3	1.8_4	1.8_2
½	58% A_1 40% A_2 Trace A_3	1.5_6	1.4_8
¾	47% A_1 49% A_2 4% A_3	1.4_3	1.3_5
1¾	36% A_1 58% A_2 6% A_3	1.3_0	1.2_0
7	94% A_2 6% A_3	0.9_4	0.9_7

FIG. 5. Changes in electrophoretic patterns as a result of dephosphorylation of ovalbumin with prostate phosphatase as a function of time (42).

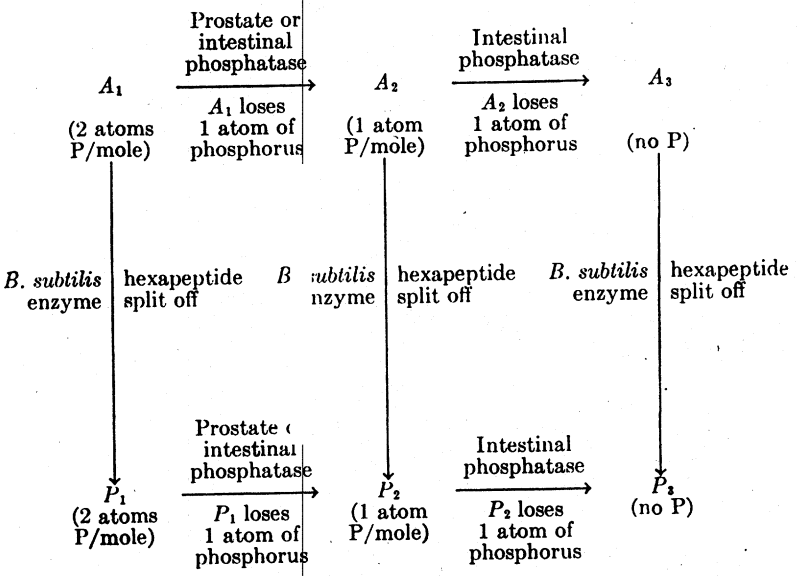
reflect a difference in the chemical binding of the two phosphate residues.

Linderstrøm-Lang and Ottesen (43, 44) have found that another enzymatic modification of ovalbumin could be isolated from protein solutions that had been stored for a long time and were found to be infected by bacteria or molds. The crystals formed by this protein were in the form of plates rather than needles. As a result, the authors called this protein plakalbumin. It was shown by these authors that ovalbumin incubated with an enzyme preparation from *Bacillus subtilis* formed plakalbumin in yields up to 90%.

Perlmann investigated the electrophoretic properties of plakalbumin and compared them to those of ovalbumin (45, 46). The mobility of plakalbumin was found to be more positive between pH 3 and 7 than that of ovalbumin and its isoelectric point to be 0.14 pH unit higher. The patterns obtained with plakalbumin showed two components P_1 and P_2 corresponding to A_1 and A_2 of ovalbumin, the phosphorus content of plakalbumin being identical with that of ovalbumin (40, 41, 44). Treatment of plakalbumin with prostate and intestinal phosphatase resulted in changes paralleling those observed in ovalbumin (41, 42), component P_2 being obtained either from plakalbumin by treatment with prostate phosphatase

or from A_2 by treatment with *Bacillus subtilis* enzyme. Eeg-Larsen and associates (47) found that the action of *B. subtilis* consisted in the splitting off of peptides, while Ottesen and co-workers (48, 49) identified the peptide as the hexapeptide alanylglycylvalylaspartylalanylalanine, split off the *C*-terminal end of ovalbumin. The over-all ovalbumin-plakalbumin transformation is summarized in Table I, and in reaction scheme I.

SCHEME I



These studies can serve as an excellent example of the combination of electrophoretic and chemical techniques in the unraveling of a complicated system. One should point out that in this case the net charge differences between individual proteins were of the order of one to two electronic charges, yet excellent electrophoretic resolution could be obtained.

A similar case of electrophoretic heterogeneity is found in ovomucoid (37, 50–53). In this case, the heterogeneity is more difficult to detect. Although at normal ionic strengths no serious heterogeneity was evident, Frédéricq and Deutsch (51) found that, if electrophoretic analyses were carried out at 0.01 ionic strength at pH 4.5, five distinct electrophoretic peaks could be observed on the ascending side. This is a good demonstration of the higher degree of resolution that can be obtained in the ascending limb at low ionic strength, which has been pointed out above. Electrophoretic patterns of this protein are given in Chapter 7. Bier and coworkers (52, 53) proved that the appearance of five peaks in electrophoresis

was due to the presence of discrete proteins by fractionation of ovomucoid by electrophoresis-convection (see Chapter 7). They succeeded in isolating O_1 in pure form and also obtained fractions enriched with respect to O_2 and O_3. The isoelectric points were found to be O_1, pH 4.41; O_2, pH 4.28; O_3, pH 4.17; O_4, pH 4.01; O_5, pH 3.83. Mobility measurements on the initial protein and various fractions indicated the absence of interactions among the various components of ovomucoid. Since this protein is known to act as a trypsin inhibitor (54), these authors tested the various fractions for this activity. Their results showed equal antitryptic activity in all the major fractions, showing ovomucoid to be a striking example of a set of electrophoretically discrete proteins originating from the same source and possessing the same biological properties.

Another electrophoretically heterogeneous, biologically active protein is the enzyme trypsin. Its heterogeneity is found, however, only under a very special set of conditions. This protein normally has electrophoretic properties typical for a "homogeneous" protein. Bier and Nord (55, 56) have shown that if the electrophoretic analyses were performed in the presence of calcium, manganese, or cadmium ions, a second minor, more slowly migrating, component appeared. These ions are known to be bound by trypsin and to stabilize it against self-digestion (57, 58). When the electrophoretic experiments were carried out in the presence of other divalent ions which have no effect on the biological activity of trypsin, no resolution into components occurred. Timasheff and associates (59) isolated the rapidly migrating component in the separation cell of the Tiselius electrophoresis apparatus and examined it for electrophoretic heterogeneity and enzymatic activity. As shown in Fig. 6, the isolated material migrates as a single component. Furthermore, no difference in enzymatic activity was found between the original protein and the pure, rapidly migrating component. It should be noted that in this case the presence of discrete closely related proteins in a single crystalline system could be demonstrated only under very special conditions, involving the specific binding of ions. Thus, one can see that great caution should be exerted in drawing conclusions on the homogeneity of a protein from electrophoretic measurements even if no splitting into components occurs over a wide pH range.

Cunningham (60) found that when trypsin is inhibited by reacting with diisopropylfluoro phosphate (DFP), the resulting material displays the electrophoretic heterogeneity shown in Fig. 6a over the pH range of 7.9 to 11.3, whether calcium ions are present or not. In the presence of calcium ions, the mobility of the major component increases, indicating binding of the divalent ions by the inactivated enzyme, as is the case with the active trypsin (61). Ram and co-workers (62) found that acetyl trypsin also displays the heterogeneity observed with normal trypsin, but the

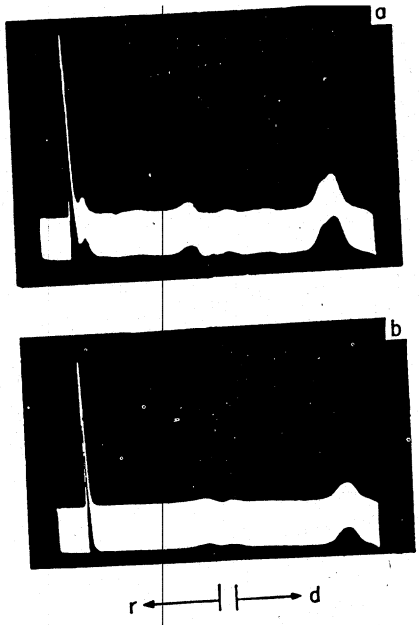


FIG. 6. Electrophoretic diagrams of trypsin preparations (59): a. Original whole trypsin. b. Rapid component after isolation in electrophoresis separation cell. Ca acetate buffer; $\Gamma/2 = 0.1$; pH = 4.7; 1800 seconds at 12 volts per centimeter.

presence of specific ions is not necessary for this. It is enzymatically active, and stable to self-digestion. It has an isoelectric point of 3.8, whereas that of normal trypsin is 10.8. It is interesting to note that in both cases of chemical modification, the electrophoretic heterogeneity becomes more easily detectable, while enzymatic activity is destroyed in one case only.

One of the most interesting recent applications of moving boundary electrophoresis has been the studies of various hemoglobins. This topic is discussed in Chapter 9. In the case of normal and sickle-cell hemoglobin, Ingram (63) has found that the two proteins differ by a single amino acid involving only one charge. And yet excellent electrophoretic resolution is obtained, showing once again the extreme sensitivity of the method of Tiselius electrophoresis for detecting very small charge differences between two proteins.

Shooter and associates (64, 65) have carried out a study on mixtures of various hemoglobins, aimed at showing the importance of the proper selection of buffer type and ionic strength for maximal resolution. They found that if a phosphate buffer of pH 6.40 and 0.04 ionic strength is used, good resolution is obtained between components in the ascending channel. Typical patterns comparing the resolution of hemoglobins A and S in

various buffers are shown in Fig. 7. The area distribution under the peaks is far from the true composition of the system, but for component identification purposes such a degree of resolution may be often desirable. The advantage of using a buffer in which ascending boundaries are artificially

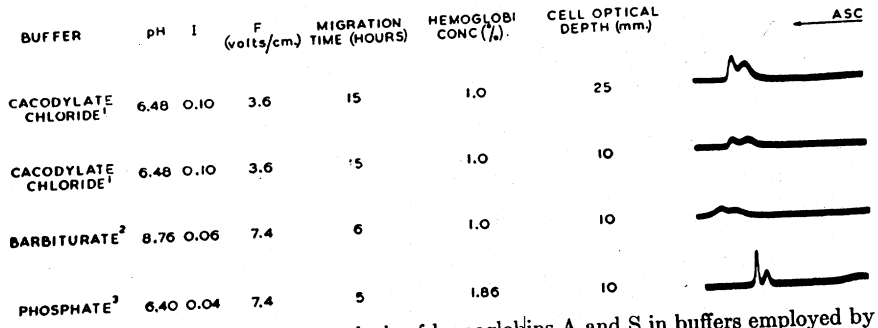


FIG. 7. Comparison of the analysis of hemoglobins A and S in buffers employed by different authors (65) [1. L. Pauling, H. A. Itano, S. J. Singer, and I. C. Wells, *Science*, **110**, 543 (1949); 2. D. B. Morrison, R. P. T. Rudnicki, and L. W. Diggs, *Federation Proc.* **13**, 267 (1954); 3. E. M. Shooter and E. R. Skinner, *Biochem. J.*, **60**, xxviii (1955).]

sharpened is further demonstrated by the complete resolution of a number of hemoglobin pairs and combinations of three in the 0.04 ionic strength phosphate buffer.

Hoch (66), in prolonged electrophoretic analysis of pepsin has shown this protein to be a mixture of four components. In this work electrophoresis was carried out with counter-compensation, and resolution into components could be obtained only after 30 cm. of migration. During short-time electrophoresis, this protein appeared to be fairly homogeneous (96% migrated as a single component at pH 3.9 and 5.9), while prolonged runs in pH 5.9 phosphate buffer of 0.025 ionic strength resulted in the resolution of the four components. The pattern was analyzed using the equation of Hoch-Ligeti and Hoch (67). It was found that, although the apparent proportion of the major component was 66%, its actual concentration is only 43%. The relative difference in migration velocities of the two main components was 1.5%. Thus, electrophoretic resolution was achieved even though the components had almost identical mobilities. Such systems can be analyzed quantitatively, however, only with the very careful application of the moving boundary theory; otherwise very large errors result in the composition analysis.

Another interesting application to a biological system is found in the work of Singer and co-workers (68), who showed the presence of two different strains of virus (tobacco mosaic virus) in electrophoretic experiments. The new strain was both detected and isolated electrophoretically.

Beaven *et al.* (69) have studied electrophoretically the difference between human fetal and adult hemoglobins, developing a method of analysis of each in the presence of the other.

A protein showing a very complex pattern of heterogeneity is β-lactoglobulin. This protein, which is the main component of milk whey, has been found by many workers to be heterogeneous below pH 5.2 (23, 28, 70–75) (its isoelectric point), while disagreement exists on its heterogeneity above this pH. The electrophoretic heterogeneity of β-lactoglobulin below pH 5.2 has been attributed by Longsworth and Jacobsen (23) to the presence of interactions and will be treated later. In the pH region between 5.3 and 6.0, Li (70) and McMeekin and co-workers (71, 72) failed to observe heterogeneity in short-duration electrophoresis experiments. Smithies (74) however, using a variety of techniques including electrophoresis, showed this protein to be heterogeneous in the same pH region. Following the discovery of Aschaffenburg and Drewry (76) that β-lactoglobulin consists of two genetically different proteins, Timasheff and Townend (28) demonstrated that its electrophoretic heterogeneity between pH 5.3 and 6.0 can be accounted for by the presence of these two proteins. In this work, prolonged electrophoretic analyses were carried out on the two individual proteins, obtained from the milk of individual cows (76), as well as on mixtures of the two proteins which Aschaffenburg and Drewry (77) designated as β-lactoglobulins *A* and *B*. The areas under the individual boundaries were found not to correspond to the correct known distribution of the two components. Therefore, a calibration graph was made using mixtures of known composition. In this way it was shown that normal β-lactoglobulin obtained from pooled milk consists of 60% *A* and 40% *B*, while the area distribution under the electrophoretic pattern gives an apparent composition of 76% *A*. This large difference between the actual composition and the area obtained on synthetic mixtures is the result of the superposition of boundary anomalies on a slight skewness of the pattern of pure β-lactoglobulin *B*. The mobilities of the two components are -0.63 (*A*) and -0.12 (*B*) at pH 5.3, and -1.67 (*A*) and -1.46 (*B*) at pH 5.6 in acetate buffers of 0.1 ionic strength. Their isoelectric points are 5.09 (*A*) and 5.23 (*B*) (78). Such differences in mobility and isoelectric points should correspond to about two charges. The electrophoretic heterogeneity of this system is complicated by the presence of protein association below pH 5.2. In the pH region above 5.2 it was shown that no association takes place (79).

In some cases electrophoretic heterogeneity can be detected only by the appearance of a shoulder on the main peak with no actual separation of components. Such is the case in insulin, which was studied by Timasheff and co-workers (80, 81). In order to prove that the heterogeneity in this

ystem is due to the presence of different proteins, insulin was fractionated
›y electrophoresis-convection (see Chapter 7). An attempt to correlate
his heterogeneity with the observation of Harfenist and Craig (82) that
nsulin contains two components, A and B, differing by one carboxylic
harge per 12,000 molecular weight, resulted in the finding that the Craig
›omponent A displays an electrophoretic heterogeneity equal to that of
vhole insulin, but a mean mobility 0.12 mobility units more positive than
he whole insulin. Since apparently the electrophoretic heterogeneity of
nsulin cannot be accounted for in terms of chemical differences, it would
seem that it is probably related to some differences in molecular folding
and hydrogen-bonding between individual molecules (83). The carboxylic
charge difference seems to play only a secondary role.

B. Boundary Spreading

In the previous discussion the components of the mixture to be analyzed could be considered as discrete. There are actually a considerable number of protein preparations which are heterogeneous but which travel as a single peak in the electrophoresis apparatus. The heterogeneity of these preparations is demonstrated best by the techniques of reversible electrophoretic boundary spreading which have been developed by Alberty and others (84–86). A detailed description of this phenomenon is given in Chapter 3. In the application of this technique, correction for diffusion is usually carried out by extrapolating the function $g(u)$ [see Chapter 3, equation (45)] to zero reciprocal time. Brown and Cann (85) however, have shown that the mobility may be expressed also in terms of the moments of the reive index gradient, X^n.

$$g(u) = \frac{1}{\beta\sqrt{2\pi}} e^{-u^2/2\beta^2} \left\{ 1 + \sum_{j=3}^{\infty} \frac{c_j}{j!} (-i)^j \alpha^j H_j \left(\frac{iu\sigma}{Et_E^2\beta^2\alpha} \right) \right\} \tag{13}$$

$$\beta^2 = \frac{\sigma^2 - \sigma_0^2 - 2Dt_E}{E^2 t_E^2} \tag{a}$$

$$\alpha = \sqrt{1 - 2(\sigma^2/\beta E t_E)^2} \tag{b}$$

$H_j(\xi)$ are the Hermite polynomials and the C_j are the coefficients of Gram-Charlier series (87). σ_0 is the standard deviation of the gradient ve at the moment the field is applied, σ is the standard deviation after trophoresis for t_E seconds, E is the electric field. The other terms are the ie as used by Longsworth (see Chapter 3, page 134). Since it is very icult to obtain accurately moments of the refractive index gradient her than the second, the usefulness of equation (13) is in general limited the determination of the second moment of the mobility distribution. number of proteins have been examined in reversible boundary-spread-

ing experiments and their heterogeneity constants have been determined. The significance of these analyses is discussed in Chapter 3 and the values of h for a number of proteins have been tabulated by Alberty (88).

The final unequivocal proof of protein heterogeneity is best provided by the isolation of the individual components. In the case where discrete components are present, fractionation can be carried out by a number of techniques. In the case of a heterogeneous system with a continuous distribution of mobilities, Cann *et al.* (89) have shown that fractionation may be carried out very successfully by means of electrophoresis-convection (see also Chapter 7). These authors fractionated bovine γ-globulin into eight fractions differing from one another by at least two electrophoretic properties, such as isoelectric point, mean mobility at pH 8.7 and mobility distribution at the mean isoelectric point. These fractions constitute a mean mobility spectrum of from $-1.25\ \mu$ to $-2.25\ \mu$ at pH 8.7 and range in isoelectric points from 7.31 to 5.74. The isoelectric point of the starting γ-globulin was 6.75 and its mobility $-1.73\ \mu$. A considerable variation in $\Delta u/\Delta$pH was found among the fractions, reflecting probable chemical and structural differences. The normalized mobility distributions of six such fractions at pH 6.5 are plotted in Fig. 8. The starting material for the preparation of fractions G and H was non-Gaussian with the result that fraction G (top) exhibited a non-Gaussian mobility distribution at its

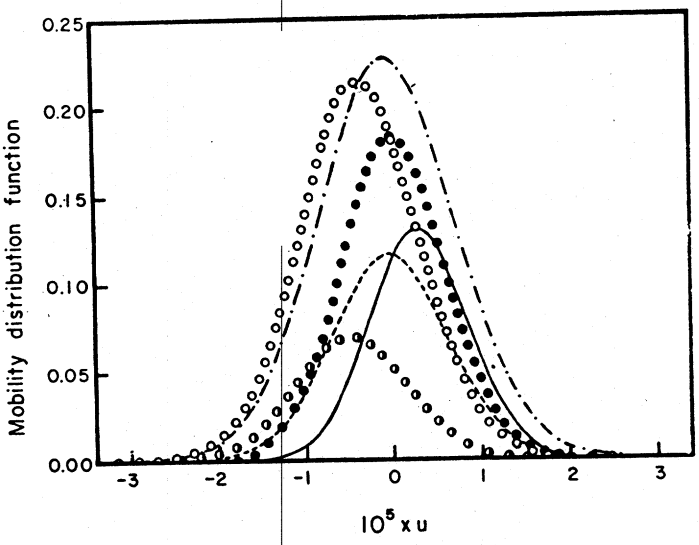


FIG. 8. Mobility distribution functions at pH 6.5 of six unique fractions of bovine γ-globulin; - - - = fraction C (pI, 6.47); ○ = fraction D (pI, 6.01); — = fraction E (pI, 7.31); ● = fraction F (pI, 6.51); ◑ = fraction G (pI, 5.74); - · - = fraction H (pI, 6.41) (89).

mean isoelectric point. This is in agreement with the theory of transport in electrophoresis-convection which predicts that the fractionation of a protein possessing a Gaussian mobility distribution will result in a top fraction which also possesses a Gaussian mobility distribution, having the same second moment as that of the original protein, while a starting material with a non-Gaussian mobility distribution will result in a non-Gaussian top fraction.

C. Microheterogeneity

In the previous two sections, a description was given of various degrees of protein microheterogeneity which can be detected by electrophoresis. In some cases, such as ovomucoid, it is possible to obtain almost complete electrophoretic resolution of the component proteins, even when the differences in physical properties are quite small. In such cases, it is often desirable to take advantage of the increased degree of resolution which can be afforded by boundary sharpening on the ascending side, even if quantitative analysis of composition has to be sacrificed. In the case of hemoglobin the difference between types A and S is a single charge, in ovalbumin the difference between the various components amount to one or two charges. One is tempted to ask what is the nature of the more subtle heterogeneity which is found in other proteins. In the case of γ-globulin, different electrophoretic fractions have been found to have different biological activities (see Chapter 7). In most cases, however, there has been no evidence associating biological heterogeneity with boundary spreading in the electrophoresis apparatus. An excellent review of the general problem of protein microheterogeneity has been written by Colvin *et al.* (90).

In some cases, the microheterogeneity has been correlated with definite chemical differences, such as in ovalbumin and hemoglobin. In the case of trypsin, heterogeneity seems to depend on the differential binding of some divalent ions by the two species. Insulin presents a case in which a known chemical difference between components (one charge per molecule) seems to be superseded by differences in secondary and tertiary structure, as evidenced by possible changes in hydrogen-bond formation shown by Frédéricq (83) and Laskowski *et al.* (91). In general, however, a continuous mobility distribution is found in a number of crystalline highly purified proteins. Part of this could be due to differences in chemical structure. For example, in serum albumin, it is known that there are two components, one of which has a free —SH group while the other one does not. It is possible that there are random variations in the spatial configuration of the nonessential parts of the protein. Such variations would affect the ionization constants of the amino acids present in the protein as shown by Tanford (18, 19). Structural variations could be of various natures.

They could be the result of differences in folding in various individual molecules, or possibly minor variations in the amino acid sequence or even composition. Furthermore, one could conceive of the existence of dynamic configurational changes in protein molecules, very slow relative to the length of an electrophoresis experiment, but sufficient to lead to the observed boundary spreading. One should also not exclude small differences between individual molecules in the effects of pH, ionic strength, and type of ions present in the system as well as the effect of the application of the electric field. Another possibility is afforded by differences in the kind and amounts of carbohydrate or hydrocarbon materials which may be tightly bound to the protein molecules. These could either contribute to the charge, or affect the folding of the molecule leading to small changes in its hydrodynamic or electrostatic properties. All these differences could lead to slight differences in electrophoretic mobilities between individual molecules. These small differences could also be exerted through variations in ion binding and molecular unfolding which might have considerable effects on the ionization of vicinal groups (18, 19). With the exception of minor differences in amino acid sequence or composition in a small number of molecules, all of these variations are in spatial configuration. It is difficult to conceive of another explanation for the microheterogeneity of proteins in view of the apparent homogeneity found by the organic chemists in all the proteins that they have studied. It is true that minor variations in a small number of molecules could go undetected by the present techniques of structural protein organic chemistry.

IV. Interactions in Protein Systems

In addition to giving information on the ionization and degree of homogeneity of a protein, Tiselius electrophoresis can yield information also on various interactions which occur in the system. The following types will be considered here: protein-ion interaction, interaction of different proteins with each other, polymerization of a protein, and isomerization of proteins. No attempt will be made to give a complete coverage of these topics, but it will be attempted to show by examples how such systems can be analyzed properly.

A. Protein-Small Ion Interactions

The binding of small ions by proteins has been studied in several cases by applying the theory of weak electrolytes in moving boundary systems. It is discussed in detail in Chapter 3, Sections IV and V. A typical example of such a study is the work of Alberty and Marvin (92) on the binding of chloride ions by bovine serum albumin, described in Chapter 3, Section V, C. Smith and Briggs (93) adapted the theory of weak electrolytes in a

somewhat different manner to a study of the binding of methyl orange by bovine serum albumin (BSA). In experiments using boundary system I of Chapter 3, Section V, A, it was found that the constituent mobility of the protein is a linear function of the amount of dye bound. In order to determine the amount of dye bound by the protein, using electrophoretic data alone, a second set of experiments using boundary system II of Chapter 3, Section V, A was carried out.

Figure 9 shows the boundary system to be expected in the descending limb. The boundary $\alpha\beta$ represents the hypothetical dye boundary. Its velocity may be determined in a separate experiment if one assumes that the protein has a negligible effect upon the mobility of the free dye. The

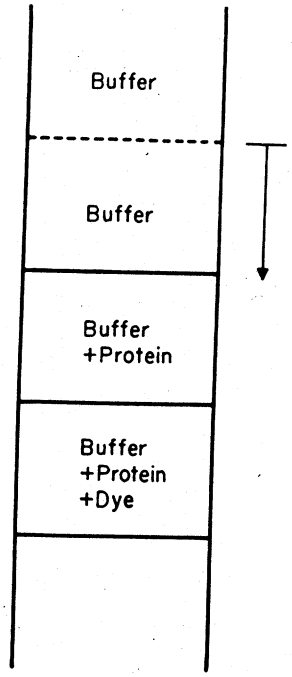


FIG. 9. Diagram of boundaries in descending limb after electrophoresis of albumin-methyl orange mixture (93).

dye disappears across the $\beta\gamma$ boundary, and its constituent mobility may be calculated from that boundary. The $\gamma\delta$ boundary gives the apparent mobility μ_p' of the protein, which differs from the true mobility of the protein, μ_p, by the factor $\kappa^\alpha/\kappa^\gamma$. All the free dye present in the original solution has passed the boundary $\alpha\beta$. Therefore, the free dye now present in β must have been bound by the protein in γ. We may set the amount

of dye originally bound by the protein in γ equal to the free dye in β. If one assumes a unit cross section for the cell, volume can be replaced by mobility differences and we get

$$[A] = [A_0] \frac{\bar{\mu}_A - \bar{\mu}_p'}{[P_0]/[P]\gamma(\mu_A - \bar{\mu}_A) + \bar{\mu}_A + \bar{\mu}_P'} \tag{14}$$

Where $[A]$ is the molar concentration of free dye; $[A_0]$ is the total molar concentration of the dye, and $[P_0]$ is the molar concentration of the protein.

$[P]^\gamma/[P_0]$ may be approximated from the areas, I, of the descending pattern and the area due to the total dye, I_{A_0}.

$$\frac{[P]^\gamma}{[P_0]} = \frac{I_{\gamma\delta}\left(1 + \frac{I_{\gamma\epsilon}}{I_{\gamma\delta} + I_{\beta\gamma}}\right)}{I_{\gamma\delta} + I_{\beta\gamma} + I_{\delta\epsilon} - I_{A_0}} \tag{15}$$

The latter can be obtained from the refractive index increment of the dye. Equation (14) enables one to calculate the amount of free dye in a protein solution from parameters measured in the electrophoresis apparatus. When the electrophoretic data were plotted by the method of Klotz *et al.* (93a), values for n (maximum number of binding sites in the protein) and K (dissociation constant) were found to be 22.0 and 2.83×10^{-4}, respectively. These are in fair agreement with dialysis experiments which have given 22.6 for n and 3.12×10^{-4} for K.

A recent electrophoretic study of the interaction of ions with serum proteins has been carried out by Schilling (94). The difference in electrophoretic mobilities in the presence and absence of cadmium, lead, silver, zinc, and sulfosalicylate ions was measured. A detailed study of the interaction of Cd^{++} ions with BSA was carried out. If it is assumed that ions are bound by n equal sites, where they are competing with hydrogen ions, the observed electrophoretic mobility change (Δu) is determined by

$$\Delta u = \frac{2\nu + \nu_H - \nu_{H_0}}{f} \tag{16}$$

where ν is the number of sites occupied by cadmium, ν_H those occupied by hydrogen ions, f is the number of charges corresponding to a unit mobility change, ν_{H_0} is the number of sites occupied by hydrogen at zero cadmium concentration. The mass action law then gives:

$$\frac{1}{f\Delta u} = a\frac{1}{[Cd^{++}]} + b$$

$$a = \frac{(1 + K_H[H^+])^2}{nK(2 + K_H[H^+])}; \; b = \frac{1 + K_H[H^+]}{n(2 + K_H[H^+])} \tag{17}$$

where K and K_H are equilibrium constants for the binding of cadmium and hydrogen ions, respectively.

A plot of $1/\Delta u$ against $1/Cd^{++}$ resulted in a straight line yielding a binding constant in agreement with that found for attachment to histidine residues.

In other studies on the binding of ions to proteins, Volkin (95) used the shift in the pH-mobility curve of insulin in the presence of thiocyanate ions, to show the binding of these ions to basic groups on that protein. Nord and Bier (56) have used similar information to show the binding of calcium ions to trypsin. The combination of detergents with proteins is another special case of small-ion binding. It has been investigated by means of electrophoresis on a number of systems. In studies on anionic detergents, Lundgren and associates (96) studied the interaction of ovalbumin with alkylbenzene sulfonates; Putnam and Neurath (97, 98) studied the interaction of serum albumin with sodium dodecyl sulfate; Ballou and co-workers (99) investigated the effect of lower fatty acid salts on the mobility of human serum albumin. Timasheff and Nord (100) investigated the interaction of a cationic detergent, dodecylammonium chloride, with ovalbumin. In all cases the mobility of the protein was shifted considerably in the presence of the detergent, indicating binding. In the presence of an excess of protein, boundaries corresponding to free protein and complex could be observed (97, 100); the free-protein boundary was found to disappear as the amount of detergent was increased, with one or more boundaries (97) due to complexes appearing. In most cases, interaction of proteins with detergents resulted in some permanent changes in the protein molecule, especially in the presence of high detergent concentration. This aspect of the problem will be discussed later.

B. Protein-Protein Interactions

The theory of protein-protein interactions is discussed in detail in Chapter 3, Sections VI and VII. A classification of various types of interactions is given there, and criteria for distinguishing between them are presented.

One of the most elegant physical studies of protein-protein interaction is the work of Singer and co-workers (101–107) on the soluble complexes of protein antigens with the corresponding antibodies.

Antibodies and antigens are substances capable of interacting specifically with one another in proportions which depend on the composition of the mixture. It has been customary to call the number of combining sites on each protein the valence of that protein. The work of Pauling and coworkers (108, 109) suggests that the precipitating antibodies are divalent and antigens are multivalent. Goldberg (110) has shown that such a system would have a zone of precipitation but that soluble complexes would exist under certain conditions, such as the presence of a large excess of antigen.

Singer and co-workers used the ultracentrifuge and electrophoresis to study the soluble antigen-antibody complexes found in the presence of excess antigen.

The antigens studied were bovine serum albumin (BSA) (101), ovalbumin (102, 103), hapten-substituted serum albumin (104, 107), chemically modified serum albumin resulting in a univalent antigen (105), β-lactoglobulin, and conalbumin.

Electrophoretic diagrams obtained on the BSA-anti-BSA system with different ratios of antigen to antibody in a pH 8.5 barbital buffer of 0.1 ionic strength are shown in Fig. 10. It is possible to see that there is no free γ-globulin present. The most rapid peak is the free antigen (BSA), while the other peaks represent complexes.

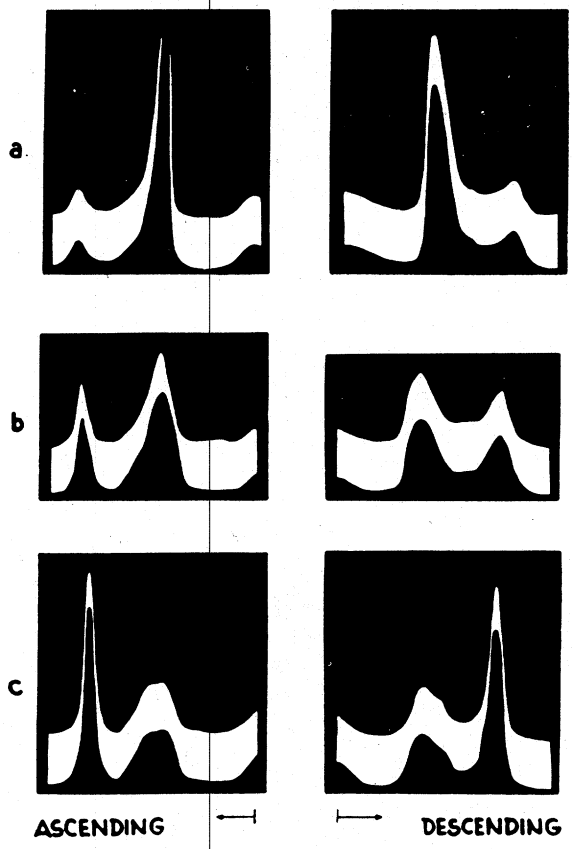


FIG. 10. Electrophoresis diagrams of soluble antigen-antibody complexes (101) at the following values of (AG/AB), total protein concentration and times of migration, respectively: a, 0.56, 18.0 mg./ml., 7740 seconds; b, 1.00; 16.1, 7800 seconds; c, 2.14, 16.5, 7560 seconds.

The complexes which exist in antibody-antigen systems constitute an equilibrium system, and the results one obtains in the electrophoresis apparatus are strongly dependent upon the rate at which equilibrium is established. Singer and Campbell (101) have set up some criteria for the quantitative use of schlieren diagrams for systems which may undergo re-equilibration.

In the ascending boundary we have Ag (free antigen) and two complexes Ag_2Ab (a complex) and Ag_3Ab_2 (b complex). The three components have mobilities $-u_A > -u_a > -u_b$. Figure 11 shows the boundary system in

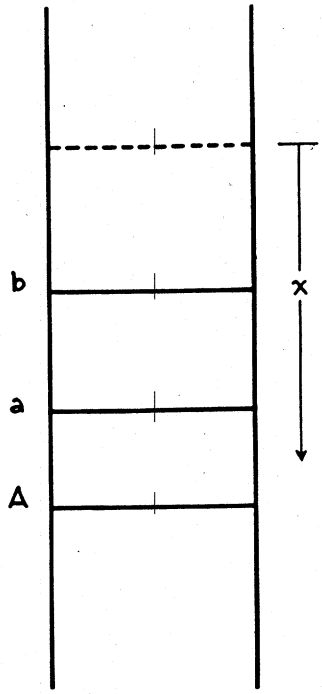


FIG. 11. Moving boundary system in the ascending limb of the Tiselius cell for the bovine serum albumin-Antibovine serum albumin system (101)

the ascending limb of the electrophoresis apparatus. In phase α behind the A boundary, there is only free antigen and no reaction takes place. In phase β, we have then the a complex and the disproportionation of Ag_2Ab may take place:

$$2Ag_2Ab \xrightarrow{R} Ag_3Ab_2 + Ag \qquad (18)$$

R is the over-all rate constant of this reaction. At some later time, the

ΔAg produced in B will overtake the a boundary and be found between A and a. The additional amount, ΔAg, of the antigen found in phase α as the result of this reaction will be

$$\Delta\mathrm{Ag}(x_1 t) = \frac{R_x x(u_a - u_b)}{(u_a - u_A)(u_b - u_A)} > 0 \qquad (19)$$

It follows that $d(\Delta\mathrm{Ag})/dx$ is positive and that the schlieren curve in the α phase will be elevated above the baseline. If, on the other hand, the schlieren curve in this region does return to the baseline, then the effects of re-equilibration are negligible and the area under the A peak on the ascending side will correspond to the concentration of free antigen present in the original solution. On the descending side, the distribution of components and the resulting shifts in equilibrium are quite different. As a result, the patterns do not return to the baseline between the peaks for the a complex and the free antigen.

Careful analysis of the electrophoretic data (101) has made it possible to obtain the equilibrium concentration of uncombined antigen in a solution of known total antigen and total antibody content. For this purpose boundary anomalies were properly taken into account by running a number of control experiments on noninteracting systems. Plotting the amount of antigen combined per antibody molecule against the relative concentration of antigen, the ratio of antigen to antibody in complexes extrapolates to a value of 2 at 100% antigen, showing the antibody valence to be 2.

An extension of these studies as a function of pH resulted in a pH-mobility curve for BSA in the complexed system identical with that of pure BSA, showing again that the effects of re-equilibration are not important. From a comparison of calculated to observed mobilities of the complexes at various pH's, further evidence was obtained for the bivalence of antibodies. Applying the Goldberg theory, equilibrium constants were calculated for the $\mathrm{Ag} + \mathrm{AgAb} \rightleftharpoons \mathrm{Ag_2Ab}$ reaction. The values obtained were $K = (2.5 \pm 0.5) \times 10^4$; $\Delta F^\circ = -5.5 \pm 0.2$ kcal.; $\Delta H^\circ = 0 \pm 2$ kcal.; $\Delta S^\circ = 20 \pm 8$ entropy units, in barbital buffer, pH 8.5 at 0°. Similar values were obtained with other proteins.

A plot of the pK for the $\mathrm{Ag} + \mathrm{Ab} \rightleftharpoons \mathrm{AgAb}$ reaction versus pH resulted in a straight line with unit slope, showing that a single ionized group is involved in every Ag—Ab bond, both in the anti-BSA and anti-ovalbumin systems.

By acetylation and guanidination of the BSA and the anti-BSA antibody, Singer (106) has shown further that there is one amino group per reactive site of the anti-BSA antibody molecule. In order to show that loss of activity is due to chemical reaction at the antibody reactive sites and not to nonspecific effects, electrophoretic experiments were carried out on a

solution of acetylated BSA-anti-BSA complex and a mixture of separately acetylated BSA and rabbit γ-globulin. The electrophoretic patterns, shown in Fig. 12, clearly demonstrate that activity is retained in the first case only.

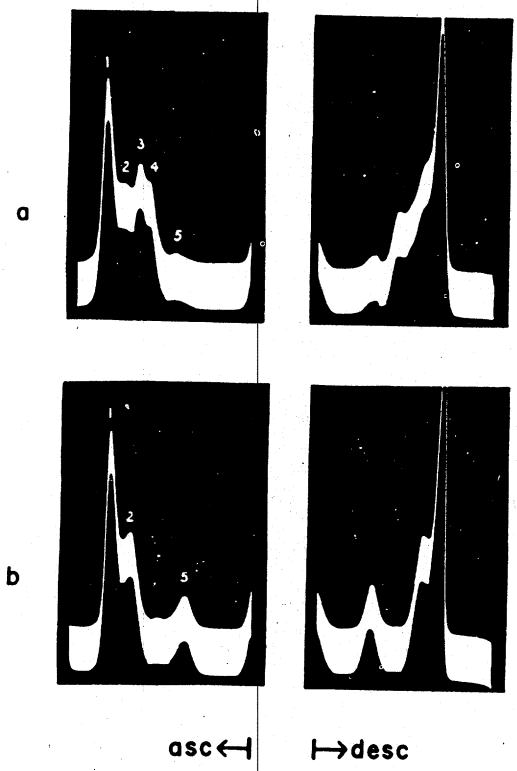


FIG. 12. Electrophoretic patterns of (a) acetylated BSA-anti-BSA complexes and (b) a mixture of separately acetylated BSA and rabbit γ-globulin in veronal buffer (pH, 8.68; Γ/2, 0.1). Boundaries 1 and 2 are due to acetylated BSA, boundaries 3 and 4 to AC-AB complexes, and boundary 5 to acetylated γ-globulin (106).

Another type of interaction between biologically important proteins is that of enzymes with specific inhibitors. Ram and associates (111) have studied electrophoretically the stoichiometry of the reaction between trypsin and its inhibitor, ovomucoid. Using an analysis of the data similar to that of Singer and co-workers, these authors found that while trypsin can bind only one ovomucoid molecule, more than one trypsin can react with each molecule of ovomucoid. The isoelectric points of the two proteins and their equimolar complex are 10.8, 4.3, and 9.0, respectively. This can be explained on the basis of the number of ionizable groups on the two proteins in this pH range. In this case also, the presence of the complexes

does not affect the mobilities of other components present in the system, and the ascending pattern returns to the baseline between peaks, showing that re-equilibration effects are not significant.

Inactivation of trypsin with the DFP reagent results in loss of its ability to complex with ovomucoid as shown by Chernikov and Shpikiter (112). Their electrophoretic data, shown in Fig. 13, are considered to be strong evidence in favor of the theory that the inhibitor is actually bound by the active site of the enzyme.

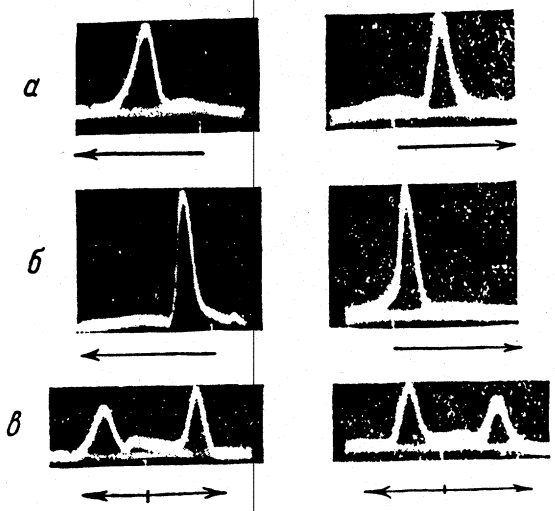


FIG. 13. Electrophoretic diagrams of the trypsin-ovomucoid system (112). a, ovomucoid; b, 1:1 mixture of trypsin and ovomucoid; c, 1:1 mixture of DP-trypsin with ovomucoid. Right: descending; left: rising. 180 Minutes in pH 7.15 veronal buffer of 0.2 ionic strength, at 3.5 volts per centimeter.

Similar data on the complexing of chymotrypsin by ovomucoid have been obtained by Weil and Timasheff (unpublished work, (112a)).

An interesting study of the complexing of two proteins has been carried out by Ehrenpreis and Warner (113) on the conalbumin-lysozyme system. In this case, the difference in the sizes of the molecules has made it possible to compare the electrophoretic data with dialysis equilibrium results. This system was found to be of the rapid re-equilibrating type (see Chapter 3, VI, A). The fact that the two proteins were oppositely charged under the experimental conditions made analysis of the data possible. The reaction was interpreted in terms of the consecutive association of two lysozyme (L) molecules with one of conalbumin (C);

$$\begin{aligned} L + C &\rightleftharpoons CL \\ CL + L &\rightleftharpoons CL_2 \end{aligned} \qquad (20)$$

The constituent mobilities, obtained experimentally as described in Chapter 3, were defined as:

$$\begin{aligned}(C)\,\bar{u}_c &= (C)\,u_c + (CL)\,u_{cl} + (CL_2)\,u_{cl_2} \\ (L)\,\bar{u}_l &= (L)\,u_l + (CL)\,u_{cl} + 2(CL_2)\,u_{cl_2}\end{aligned} \tag{21}$$

The corresponding equations for the constituent concentrations are:

$$\begin{aligned}(\overline{C}) &= (C) + (CL) + (CL_2) \\ (\overline{L}) &= (L) + (CL) + 2(CL_2)\end{aligned} \tag{22}$$

where the u_i's are the mobilities of the corresponding species. Since at the experimental pH, conalbumin and lysozyme have about equal but opposite charges, u_{cl} is small, and since (CL_2) is also small, $(CL)\,u_{cl} + (CL_2)\,u_{cl_2}$ in equation (21) could be neglected. This made possible the analysis of the electrophoretic data, which turned out in good agreement with dialysis equilibrium results. In cases in which the last two terms in equation (21) are sufficiently large so that they cannot be left out, quantitative analysis becomes impossible, since there are more unknowns than equations.

The system ovalbumin-yeast nucleic acid (114) has also received a detailed quantitative study. This is discussed in Chapter 3, Section VI, B. Similar studies have been carried out on the interaction of ovomucoid with yeast nucleic acid (114), of serum albumin with thymus nucleic acid (115) and with fish sperm nucleic acid (116), as well as of insulin with protamine (117). Longsworth and co-workers (37) have pointed out the interaction of various components of egg white. Mixtures of α- and β-casein (118), as well as of the various components of gliadin (119), also display electrophoretic evidence of interaction.

C. Polymerization Reaction

A number of systems exist in which the protein aggregates reversibly under a given set of conditions. For the case in which the re-equilibration is rapid, Gilbert (120) has shown that for the reaction $nB \rightleftharpoons B_n$, $K = (B)^n/(B_n)$, if n is greater than 2, the refractive index gradient curve may have two maxima. For a dimerization reaction only one boundary will be present. Using this theory, which is discussed in detail in Chapter 3, Section VII, B, it is possible to determine the equilibrium constant of the reaction, if the extent of aggregation (n) is known.

A number of protein systems are known which undergo a polymerization reaction. Among those that have been studied electrophoretically are arachin (121), chymotrypsin (122), and chymotrypsinogen (122). The most thoroughly studied polymerizing system is β-lactoglobulin (23, 72, 75,

123–125). Ogston and Tilley (75), in comparing electrophoretic experiments with some ultracentrifuge measurements showed that the electrophoretic heterogeneity of that protein at pH 4.65 is due to polymerization. Townend and Timasheff (79) showed this association to be limited to the pH region between 3.5 and 5.25 with maximal association taking place between pH 4.40 and 4.65. Following the discovery by Aschaffenburg and Drewry (76) that this protein consists of two genetically different entities, Ogston and Tombs (123) found that the electrophoretic patterns of the two are different in the pH region of association, with the conclusion that β-lactoglobulin *A* probably associates to a much greater extent than β-lactoglobulin *B*. A comparison of electrophoretic and ultracentrifugal patterns for these two proteins was carried out by Townend and Timasheff (125). Typical patterns are shown in Fig. 14. It can be seen that, whereas both

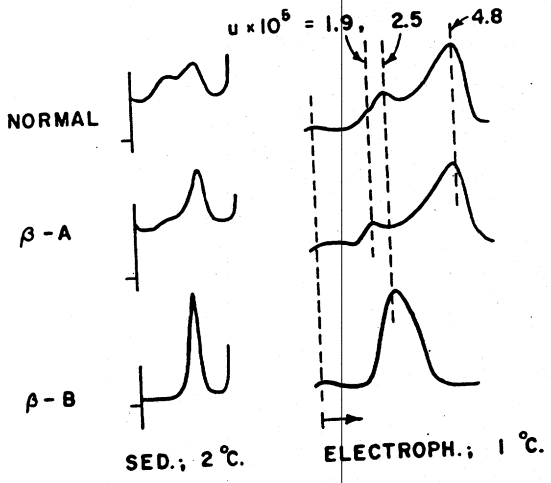


FIG. 14. Tracings of ultracentrifugal and electrophoretic patterns (descending) of various β-lactoglobulins in pH 4.65 acetate buffer, Γ/2 = 0.1 (125). Both sedimentation and electrophoretic migration proceed from left to right. Sedimentation: 59, 780 r.p.m.; "normal" and β-A, 1.4% protein, 160 minutes; β-B, 7% protein, 352 minutes. Electrophoresis: 1.6% protein, 8000 seconds at 9.7 volts per centimeter.

proteins give complex electrophoretic patterns, only *A* shows evidence of association in the ultracentrifuge.* Light-scattering studies by Townend and Timasheff (125) have shown that the re-equilibration is very rapid (less than 1 minute) and that the polymer formed is most probably a tetramer. Using this information, as well as Tombs' report (124) that only 90% of the A protein can give the complex electrophoretic patterns, Townend and Timasheff calculated an equilibrium constant for the re-

* Klostergaard and Pasternak (78) have come to the opposite conclusion.

action $4\beta^{A'} \rightleftharpoons \beta_4^{A'}$ with the aid of the Gilbert theory (120). The equilibrium calculations were done in the following manner. For $n = 4$, the function δ at the minimum point of the pattern is $\delta = (n - 2)/(3n - 3) = 0.222$. From the experimental data on the area distribution at a series of protein concentrations, one can calculate the concentration of polymerizable protein at which 50% will be aggregated, since above that concentration the fraction of the slow peak due to the polymerizable protein must have a constant area at all concentrations (120). In the case of β-lactoglobulin A, when correction of the areas was made for 10% nonpolymerizable material, it was found that the slow peak at the concentrations studied corresponded to 0.80 ± 0.20 gm. per liter of protein monomer. A comparison of calculated and observed area distributions under the two peaks of the reaction boundary on the descending side is given in Table II.

TABLE II

ASSOCIATION OF β-LACTOGLOBULIN A[a,b]

Protein conc. g/l	% Major component	
	Observed	Calculated[c]
5.2	71	74
10.0	79	82
16.0	86	85
16.3	85	85
20.4	87	86
12.8[d]	91	93

[a] According to Townend and Timasheff (28).

[b] 90% aggregable material (124). Area distribution in electrophoresis in pH 4.65 acetate of 0.1 ionic strength, 1°C.

[c] Calculated according to Gilbert theory (120); $K = 7.8$ gm.^{3} -1^{-3}; $n = 4$.

[d] Purified aggregable fraction.

Using the value of 0.80 gm. per liter for the constituent concentration, $\bar{m}$, at the minimum point and the corresponding δ value for $n = 4$, K was found to be equal to 7.8 ± 3.4 gm.^{3} $- 1^{-3}$. This results in a standard free energy of association of $\Delta F^\circ = -15.4 \pm 0.5$ kcal. per mole at 1° for the tetramerization of the polymerizable fraction of β-lactoglobulin A. These values of K and ΔF° are in good agreement with corresponding values obtained from light scattering and ultracentrifugal data. It might be of interest to note that application of the Gilbert theory to ultracentrifugal data on the polymerization of β-lactoglobulin at pH 4.65 (125), as well as to its dissociation into half-molecules at pH 1.6 (126, 127), made it possible to

calculate K values in good agreement with light-scattering data, as well as the concentration dependence of the sedimentation constant.

D. *Isomerization Reaction*

In Chapter 3, Longsworth described qualitative criteria for recognizing boundaries in which re-equilibration of isomers occurs. Cann and associates (128) have made a theoretical study of the electrophoretic behavior of systems containing a protein which can exist in two interconvertible states, A and B.

In treating the problem, it was assumed that the states A and B have mobilities μ_1 and μ_2 and diffusion constants D_1 and D_2. The specific rates of interconversion are k_1 and k_2,

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \tag{23}$$

and the concentrations of A and B are represented by C_1 and C_2. During electrophoresis of this system, the changes in concentrations with time of electrophoresis, t, and height in the electrophoresis cell are described by the following set of forced-diffusion equations where the position variable, x, is measured relative to a frame of reference moving at velocity $\mu_1 Et$ and E is the field strength.

$$\text{(a)} \qquad \frac{\delta C_1}{\delta t} = D_1 \frac{\delta^2 C_1}{\delta x^2} + k_2 C_2 - k_1 C_1$$

$$\text{(b)} \qquad \frac{\delta C_2}{\delta t} = D_2 \frac{\delta}{\delta x}\left[\frac{\delta C_2}{\delta x} - \frac{\gamma E C_2}{kT}\right] - k_2 C_2 + k_1 C_1 \tag{24}$$

$$\text{(c)} \qquad \gamma = \frac{z_2 e \mu_2 + z_1 e \mu_1}{\mu_2}; \; \mu = ze\mu; \; D = nkT$$

The symbol z represents the net electrical charge and e the electronic charge.

If the concentrations are represented by their Fourier integrals,

$$C(x,t) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} G(\sigma,t) e^{-i\sigma x} \, d\sigma \tag{25}$$

the equations (24) can be solved for G_1 and G_2. C_1 and C_2 may then be obtained by evaluation of the Fourier integral (25). Analytical solution of the Fourier integrals for the general case is extremely difficult. However, an approximate analytical solution can be obtained for the special case where $k_1 = k_2 = k$ and $D_1 = D_2 = D$. If it is assumed that k is sufficiently large, so that $4k^2 \gg (\sigma\gamma' E)^2$ where $\gamma' = \mu_2 - \mu$, then the electrophoretic gradient curve is given by the relation

$$\frac{-\delta(C_1 + C_2)}{\delta x} = \frac{C_{20}}{\sqrt{\pi}} e^{-(\frac{1}{2}\gamma' Et - x)^2/4Dt}$$

$$\left\{\frac{1}{(Dt)^{1/2}} - \frac{\gamma'^2 E^2 e^{-2kt}}{32k^2(Dt)^{3/2}} + \frac{\gamma'^2 E^2 e^{-2kt}}{64k^2(Dt)^{5/2}}(\tfrac{1}{2}\gamma' Et - x)^2\right\} \quad (26)$$

where C_{20} is the concentration of component B at zero time. It can be seen by inspection that in the limit of infinitely large rates of reaction, where equilibrium would be expected to be maintained during electrophoresis despite differential migration of the components, equation (26) predicts that the system as a whole will migrate as a homogeneous substance. On the other hand, bimodal boundaries are predicted for rather slower rates of reaction.

Some illustrative calculations have been made using equation (26) expressed in terms of the reduced variables $x' = x/l$, $t_0 = kt$ and $\alpha = D/kl^2$ where l is a characteristic length equal to $\gamma' E/k$. Theoretical gradient curves at several values of the reduced time, t_0, for each of two values of k are shown in Fig. 15. These calculations indicate that resolution into two

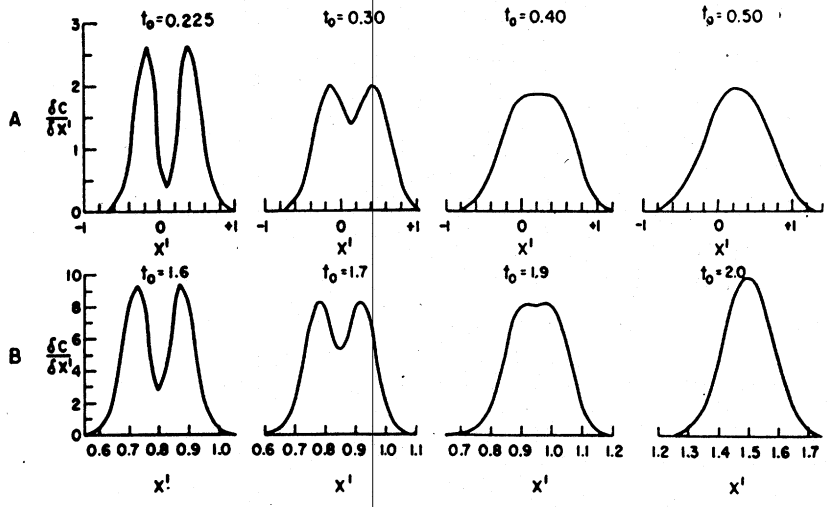


FIG. 15. Illustrative calculation of electrophoresis gradient curves using equation (26): A, $\alpha = 10^{-1}$; B, $\alpha = 10^{-3}$ (128).

boundaries will occur for times of electrophoresis less than, or of the order of, the half-time of reaction, but that for longer times a single moving boundary spreading by diffusion only will be observed.

The theory of isomerization equilibrium in electrophoresis presented above is obviously correct only within the limits of its assumptions. It

should provide a valuable guide in the interpretation of electrophoretic data. However, its limitation should be recognized. It does not discuss the possibility and consequences of pH, salt, and density gradients which may exist across the boundaries. The more complicated reactions involving more than one molecule are beyond the scope of the theory.

E. The Behavior of Proteins in Acid pH's

The subject of equilibrium systems in electrophoresis has been receiving considerable attention lately because of the interest in the behavior of proteins below pH 4.5. While a number of protein systems have been examined, bovine serum albumin has become more or less the standard, and most of the studies have been done with this protein.

Bovine serum albumin normally travels as one peak in the electrophoresis apparatus above pH 4.5. Below this pH more than one peak is observed and the patterns are usually nonenantiographic. Furthermore, the pH range of electrophoretic heterogeneity is found to be a function of the buffer used.

The first observation on the electrophoretic heterogeneity of serum albumin was made by Luetscher (129), who found that crystalline human and horse serum albumins show two electrophoretic boundaries in acetate buffer of pH 4.0 and ionic strength 0.02. Sharp and co-workers (130) reported that horse serum albumin displayed a complex boundary pattern in the pH range between 4 and 6 both at 0.1 and 0.02 ionic strength. These patterns were distinguished by a lack of symmetry between the ascending and descending boundaries, which was particularly pronounced at pH near 4.4. Longsworth and Jacobsen (23) concluded that the nonenantiographic patterns of this protein indicate continuously readjusted equilibria across the moving boundaries. The heterogeneity of serum albumin in acid pH's has been observed also by a number of other workers (131–133).

The problem of the heterogeneity of BSA in acid pH's recently has undergone broad investigations by Aoki and Foster (134–139) and by Cann and Phelps (140–143).

Aoki and Foster have examined the electrophoretic behavior of BSA in the pH range of 2 to 5 using 0.2% protein solutions at 0.02 ionic strength of chloride, thiocyanate, and acetate. In the pH region between 3.0 and 4.5, two boundaries were observed, the percentage composition changing continuously with pH. At 0.2% protein, the patterns were found to be fairly enantiographic, while at higher concentration, enantiography was lost. These authors explained the anomalous behavior of bovine serum albumin in the electrophoresis apparatus at pH 4.0 by an isomerization reaction of serum albumin involving a change in the intrinsic ionization constants of the carboxyl groups of the protein. The observations were explained in

terms of the equilibrium

$$N + 3H^+ \rightleftharpoons F$$

The titration data for BSA are also anomalous below pH 4.5 (144). Aoki and Foster accounted for the titration data obtained between pH 3.5 and 4.5 by assuming that the carboxyl groups of the two forms have different intrinsic ionization constants, with the N form having a pK_0 value of 3.7 and the F form, 4.4. Assuming that the area under the fast-moving peak in the electrophoresis apparatus represents the concentration of the F form and that the area under the slow-moving peak represents the N form, Aoki and Foster calculated the pH-dependence of the distribution of the two forms. This is shown in Fig. 16, and is in good agreement with their interpretation of the titration data.

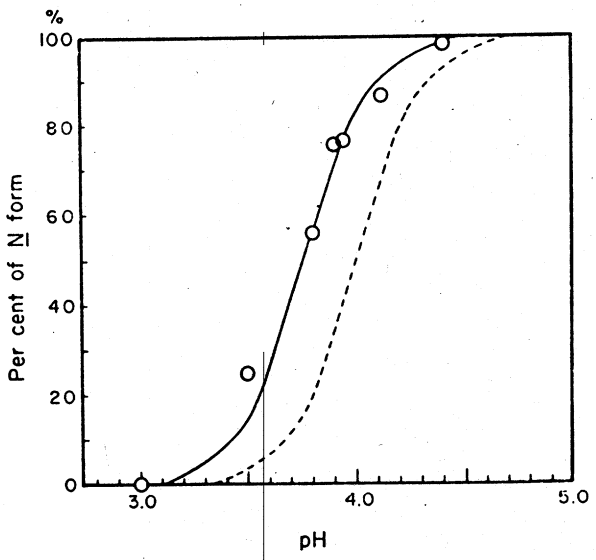


FIG. 16. Dependence of electrophoretic composition of bovine serum albumin on pH at 0°C., 0.2% protein. — 0.02 ionic strength SCN^-; - - - 0.02 ionic strength Cl^- (136).

Further investigations of this problem under the same conditions, as well as in the presence of urea (138) and detergent ions (139), have given evidence that the composition and the mobilities of the components are constant over a wide variety of lengths of runs, indicating re-equilibration to be not important. In the interpretation of the data, however, Foster and Aoki had to assume that the equilibrium represents the sum of four cooperative transitions (137) in order to reconcile quantitatively the electrophoretic and titration information.

It has not been established unequivocally that the electrophoretic patterns obtained with BSA in the pH region of 3.5 to 4.5 can be interpreted in this simple manner. Certain difficulties are yet to be overcome. Correlation of the titration data with electrophoresis requires knowledge of ion binding. Unfortunately, the information on the binding of chloride and thiocyanate ions as a function of pH is as yet incomplete. Furthermore, the assumptions of four cooperative transitions are yet to receive independent experimental validation. One should also point out that a serious discrepancy exists between the experimental reaction velocity and the predictions of the theory of Cann *et al.* (128). Thus, until further experimental and theoretical developments become available, the above interpretation of the data will remain open to question and should be considered only as a very interesting model for such protein behavior. It should be pointed out that in a recent publication, Bro and Sturtevant (145) have reported on calorimetric evidence showing that a transition is occurring in BSA in the pH region of 3.5 to 4.5, with a heat of reaction similar to that calculated by Aoki and Foster (135) from electrophoretic data and a half-time of about 2 minutes. Furthermore, Tanford and associates have concluded from titration and viscosity data that below pH 4.3, BSA undergoes a change from a compact to an expandable form (144).

Cann and Phelps (140–143) have carried out similar studies of the electrophoretic behavior of BSA, ovalbumin, and γ-globulin. Working at pH 4.0 in low and high ionic strength buffers, at protein concentrations of approximately 1.3%, these authors have observed that the three proteins display complex nonenantiographic patterns. (The work with γ-globulin was carried out at pH 5.7 and 4.0.) Electrophoretic patterns obtained in acetate-chloride buffers of various compositions are shown in Fig. 17. As can be seen, progressive changes in the electrophoretic patterns occur as the concentration of NaAc-HAc increases. These were found to be very sensitive to the NaAc-HAc concentration, but not to ionic strength, if the acetate concentration remained constant. Comparison of data obtained in a series of carboxylic acid buffers showed a close correlation with the concentration of nonionized carboxylic acid in the supporting medium and a very poor one with the acid anion. A similar study in the presence of formic acid showed the electrophoretic patterns to be strongly pH-dependent, suggesting the participation of carboxylate groups on the protein in this reaction. This is consistent with the finding of these authors that the electrophoretic patterns of methylated BSA at pH 4.0 are insensitive to the concentration of the acetate buffer (145a).

A tentative qualitative interpretation of this behavior can be given in terms of the binding of undissociated acid molecules by the protein with

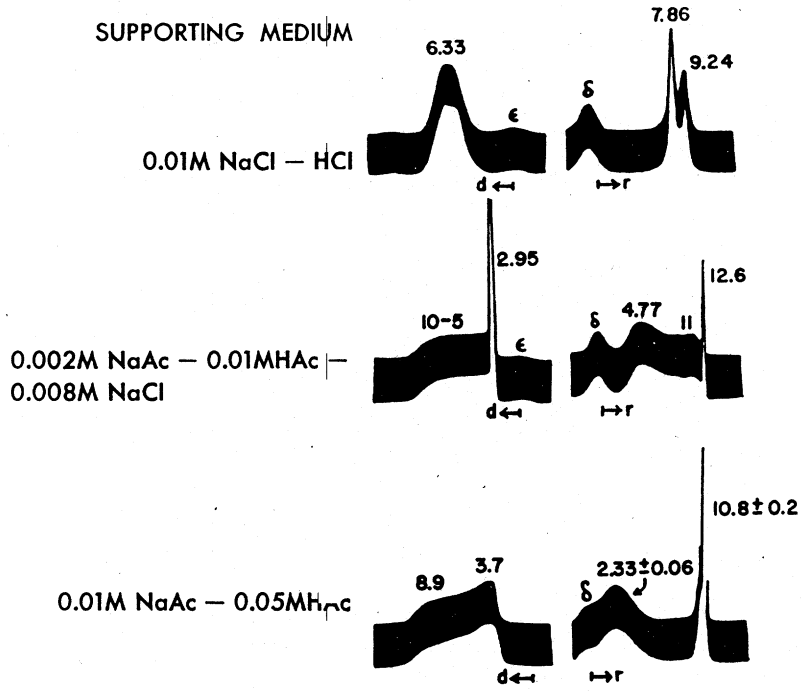


FIG. 17. Electrophoretic patterns of 1% BSA in various supporting media at pH 4.0. Boundary velocities ($10^5 \times u$) are shown above or beside corresponding peaks (143).

the assumption that the resulting complex has a higher mobility than the original protein (145a):

$$P \text{ (slow)} + \text{HAc} \rightleftharpoons P\text{HAc (fast)}$$

The electrophoretic pattern can then be explained with the help of the boundary diagram shown in Fig. 18. On the descending side, the *P*HAc form moves with its normal mobility into the original equilibrium mixture, leaving behind *P*. In order to readjust equilibrium, *P* reacts with HAc from the buffer, lowering the HAc concentration behind the *P*HAc boundary and raising the pH in that region. This causes a retardation in the migration at the front of the slow *P* boundary and results in sharpening, since the rear portion of the boundary migrates in the original lower pH buffer. The descending limb should then consist of a leading broad *P*HAc boundary, followed by a sharp slow boundary, with the pH between the two higher than the original pH. Separation into two boundaries may occur even if the reaction is very rapid as shown by Gilbert and Jenkins (146). On the rising side, the rapidly migrating *P*HAc moves out into fresh buffer. Due to re-

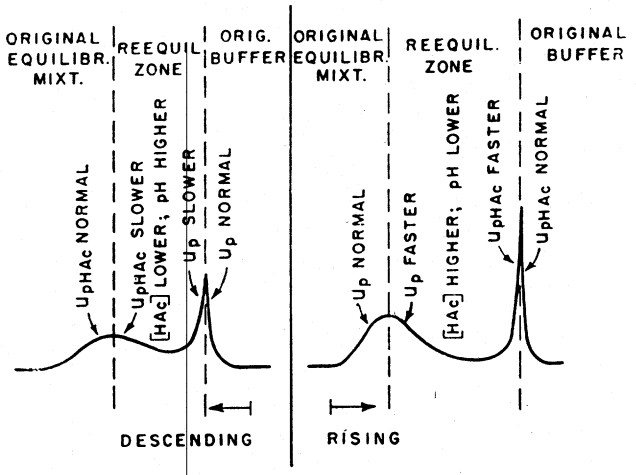


FIG. 18. Model boundary system that could account for effects of acetic acid binding on electrophoresis of BSA in pH 4.0 acetate buffer.

equilibration, HAc is liberated behind the boundary, lowering the pH behind the boundary and causing its sharpening. The boundary, due to slower-migrating P, moves into the zone of lower pH, its leading edge becoming accelerated, while its tailing edge migrates in the original buffer. This would result in a pattern consisting of a sharp, rapid boundary, followed by a slow, broad one, with the pH between the two being lower than in the original solution.

Cann has measured the actual pH and conductivity at various points on the rising side and compared these values with those calculated from the Dole theory. It is of interest that while the changes in conductivity, observed behind the various boundaries, can be accounted for in terms of the Dole theory, the pH changes were always found to be significantly greater than those calculated and in qualitative agreement with those expected from the above-described mechanism. Thus, it would appear that at higher protein concentrations, although protein disappears across the various moving boundaries, these may be considered as false boundaries in the sense that they correspond neither to single stable protein components nor to single components involved in a slowly adjusted equilibrium. These false boundaries do not arise solely as a result of the establishment of conductance and pH gradients in the electrophoresis cell, but are associated with complexing of the protein with buffer constituents, in particular with the undissociated buffer acid.

Phelps and Cann (142) have found further that introduction of amino acids into the buffer system results in changes that could be interpreted

as competitive binding of the amino acid to the same sites as the carboxylic acid. The active form in this case is the acid form of the amino acid rather than the zwitterion, and steric factors are important in its action.

Since the theory of the effect of binding of neutral molecules on the electrophoretic behavior of a protein has not been developed yet, a complete understanding of these observations is not possible.

Observations, similar to those described above, have been made by Singer and Campbell (101), who have found that in glycine-HCl buffers of pH 2.35 and 0.1 ionic strength, BSA, γ-globulin, β-lactoglobulin, ovalbumin, and conalbumin all give two peaks in both sides of the electrophoresis cell. Experiments were carried out on BSA by Dintzis, Timasheff, and Singer (146a) in which the rapid peak was isolated from the rising side of the Tiselius cell and the slow peak from the ascending side. Subsequent electrophoresis at the same conditions of the two isolated "components" showed a picture identical with the original pattern no matter how rapidly the sample was removed and rerun. This observation is identical with those of Cann and Phelps (141, 142) on BSA and ovalbumin at pH 4.0, who also obtained patterns identical with the original material on rerunning samples withdrawn from the Tiselius cell.

Recently, the problem of protein heterogeneity in the presence of amino acids has been extended by Woods (147), who examined BSA, ovalbumin, lysozyme, bovine γ-globulin, bovine fibrinogen, a water-soluble gelatin, and poly-L-lysine in various buffers between pH 1.5 and 3.0. In all cases, Woods found that when the buffer contained an amino acid hydrochloride, patterns similar to those observed by Singer and Campbell (101) were obtained, while normal patterns were found in other buffers. Analysis of this phenomenon led to the conclusion that the behavior of these boundaries does not fit the requirements of a false boundary of the Svensson type (26). Woods (147) suggests that the patterns can be interpreted qualitatively in terms of the moving boundary theory of weak electrolytes. It is of interest, however, that, if it is assumed that the glycinium form of the amino acid is bound to the protein with the formation of a more rapidly migrating species, it is again possible to account qualitatively for this behavior in terms of the boundary pattern used with BSA in the presence of NaAc-HAc (Fig. 18). Indeed, the pH changes expected across the various boundaries in such a system are in qualitative agreement with those determined by Woods (147) in both the rising and descending limbs of the cell.

Nonenantiographic patterns similar to those of Cann and Phelps, have been observed on BSA by Schilling (94) in 0.1 ionic strength NaAc and cadmium acetate buffers at pH 6.0.

A very interesting finding is that of Schmid (148), who prepared a serum albumin which gave normal patterns in acetate buffer at pH 4. After treatment with cysteine and an ion exchanger, however, nonenantiographic patterns, similar to those described above, were obtained. Schmid suggests that cysteine removed a blocking agent from the protein (148), permitting it to enter into the isomerization and acid-binding reactions described above.

The question of the exact nature of the equilibrium mixtures observed in the electrophoresis apparatus at low pH is still open. Recent experiments by Cann (143) on the effect of protein concentration on the electrophoretic patterns of BSA in 0.01 Ac^- 0.01 Cl^- at pH 4.0 indicate that the effects, observed by Aoki and Foster (134–139) and by Cann and Phelps (140–143), are different. It was found that the slow boundary, observed by Cann and Phelps, arises gradually as the protein concentration is increased from 0.2 to 1%. As shown in Fig. 19, at 0.2% only the two components studied by Aoki and Foster are present, while at 1%, the pattern becomes much more complex. Thus, in the present state of knowledge, one might make the

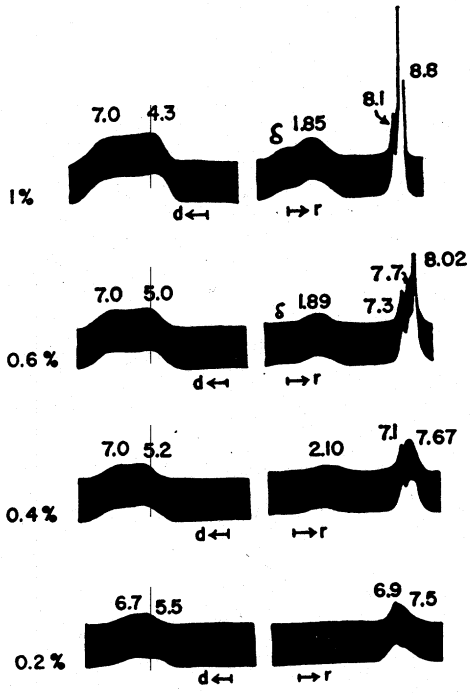


FIG. 19. Electrophoretic patterns shown by BSA at various protein concentrations in 0.01 *M* NaAc, 0.05 *M* HAc, − 0.01 *M* NaCl, pH 4.0. Boundary velocities ($10^5 \times u$) are shown above or beside corresponding peaks (143).

tentative conclusion that in the pH region close to 4, two distinct phenomena occur in BSA; one is a transformation of the protein, best observable at low concentration, the other is binding of buffer acid, which leads to complex electrophoretic patterns.

The interpretation of the electrophoretic data might be somewhat easier if more information were available on the pH and conductivity changes across the boundaries in the various systems studied. It seems probable, however, that the observed electrophoretic behavior of proteins in acid pH's is the result of the cooperative influence of true heterogeneity, isomerization, and the binding of ions and small molecules. Thus, a great deal of work remains to be done before this question is fully elucidated. The mechanisms presented above can serve as possible hypotheses that might account for the observations. In no case should they be considered as final interpretations of the data.

F. Protein Interactions

While the theories of various types of reactions that can occur in proteins are far from completely developed, a great deal of progress has been made in the understanding of patterns found in reacting systems. Only a few reaction cases are known where the pattern is enantiographic. These are the protein-detergent systems (96, 97, 100), the antigen-antibody system (101–105), the trypsin-ovomucoid interaction (111, 112) and the BSA study in acid pH at low concentrations (134, 135, 138, 139). In some of these cases the boundaries may be interpreted as representing single species of protein or complex, and their areas and mobilities can be considered as a first approximation to correspond to those of discrete components. In most cases, however, the patterns are strongly nonenantiographic and great care has to be exerted in their proper interpretation. A great deal more of theoretical work is necessary, however, especially on the effects of salt, pH, and density gradients on the reaction boundaries, and on the binding of neutral molecules to proteins, as well as on the moving boundary theory of weak electrolytes, before a complete understanding of electrophoretic systems in equilibrium can be reached.

As a further word of caution on the interpretation of electrophoretic patterns obtained in interacting systems, it must be pointed out that many of the proteins, which are considered to be "pure," have been found recently to be resolvable into several components by various techniques, such as chromatography. As an example, it is sufficient to cite the work of Tiselius and associates, who separated BSA into three distinct components (149). Since various components of a "pure" protein may exhibit different behavior in interactions and isomerizations, the actual systems may be much more complicated than is realized at present. One can cite as ex-

amples the electrophoretic difference at pH 4.0 between BSA as prepared by Schmid and standard BSA preparations, as well as the difference in association properties of β-lactoglobulins *A* and *B*.

V. Modification of Proteins

Electrophoresis can be useful, also, in the detection and characterization of permanent changes in protein molecules. These can be of various natures. They may be the result of chemical or enzymatic treatment, of changes in the physical environment, or of storage under even mild conditions. In some cases, chemical modifications have been applied very successfully in obtaining an insight into the mechanism of the biological activity of a protein.

A. Chemical Changes

A typical example of small chemical changes induced by enzymatic action that has been studied in detail electrophoretically is the ovalbumin-plakalbumin system (42–49). Here, as shown in Section III, A, a combination of chemical and electrophoretic analyses led to an understanding of the system. Another important case that has been discussed above is Singer's work on the antigen-antibody system (106) in which chemical blocking of particular groups with subsequent electrophoretic analysis led to the identification of an ϵ-amino group as being essential for the formation of the Ag-Ab bond (Section IV, B). In the case of enzymes, reaction of trypsin with DFP led to a change in its heterogeneity pattern as well as its capacity to bind a specific inhibitor (60, 112), while acetylation resulted in a similar change in the electrophoretic heterogeneity pattern (62), which in normal trypsin is contingent on the binding of some specific ions; in the last case, however, there is no loss of the ovomucoid binding capacity, nor of enzymatic activity (Section III, A). A few other types of studies that have been carried out will be mentioned briefly, while no attempt will be made to cover the subject completely.

Dreyer and Neurath (150) and Pechère and Neurath (151) have used Tiselius electrophoresis in very elegant studies on the rates of activation of chymotrypsinogen and trypsinogen, respectively.

In Fig. 20 are given electrophoretic patterns of the chymotrypsinogen-chymotrypsin transformation system (150). The electrophoretic pattern of chymotrypsinogen indicated that protein to be at least 97% homogeneous. During the activation, as can be seen on Fig. 20, the boundary corresponding to chymotrypsinogen was gradually replaced by components of decreasing mobility identified as π- and δ-chymotrypsin. At intermediate times of activation, the π-enzyme predominates, while at the end of 90 minutes of activation, at least 90% of the protein had a mobility charac-

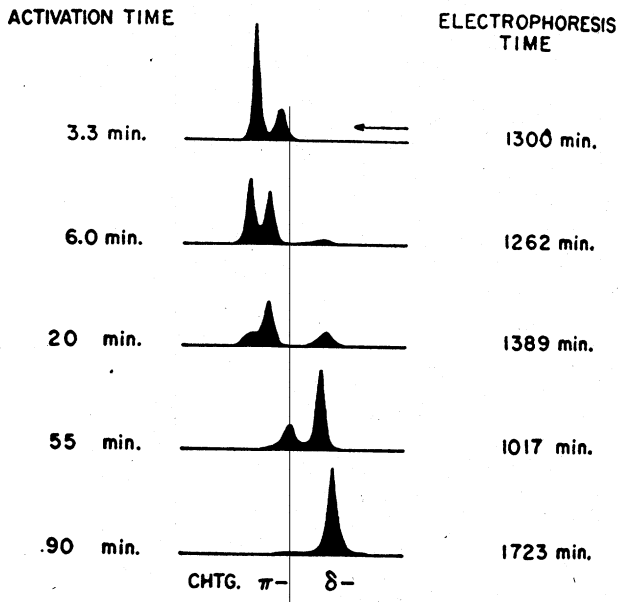


FIG. 20. Ascending electrophoretic patterns of chymotrypsinogen (CHTG.) activation mixtures. Acetate buffer, pH, 4.97, Γ/2, 0.1; 5.8 volts per centimeter. The ascending mobilities are: chymotrypsinogen, 3.8×10^{-5}; π-chymotrypsin, 3.65×10^{-5}; δ-chymotrypsin, 3.25×10^{-5} cm²-volt⁻¹-sec.⁻¹ (150).

teristic of δ-chymotrypsin. In this manner, it was possible to follow the kinetics of formation and disappearance of the various components which take part in this transformation.

A similar study was carried out on the trypsinogen-trypsin system (151). In this case, a complication arose due to the fact that the trypsin had to be inhibited as it was formed. Therefore, the research was carried out in the presence of soybean trypsin inhibitor. After accounting properly for the contribution of the inhibitor to the area on the electrophoretic patterns, Pechère and Neurath were able to obtain a curve for the rate of formation of trypsin which agreed quantitatively with data obtained from activity measurements.

Free electrophoresis has been used extensively in the study of the interaction of proteins with detergents. These have been mentioned above (Section IV, A) and reviewed thoroughly by Putnam (98). In all cases studied, the protein was altered to some extent. An interesting example is the work of McMeekin and co-workers (152), who isolated a crystalline complex of β-lactoglobulin with dodecyl sulfate, containing two equivalents of detergent per molecule of β-lactoglobulin (mol. wt. 35,000). Electrophoretic data in pH 4.8 acetate buffer of 0.1 ionic strength resulted in a

single slightly skewed peak of reduced mobility, while in pH 8.4 barbital of 0.1 ionic strength the mobility of the complex was more negative than that of the native protein. In that pH region, titration data showed the acid-binding capacity of the complex to be two equivalents lower than that of the native protein. At pH's close to 4, the titration curves were approximately the same. The observation would seem to indicate that binding of the detergent has a strong effect on the association reaction at pH 4.8 (see Fig. 14).

Recently, Foster and Aoki (139) have reinvestigated the interaction of bovine serum albumin with sodium dodecyl sulfate and interpreted the data in terms of the proposed *N-F* isomerization of this protein (see Section IV, E) (134–139). It was found that the detergent has a pronounced effect in displacing the equilibrium toward the *N* form. The dependence of the electrophoretic composition on detergent ion concentration could be fitted most accurately by assuming that the *N* form possessed ten strong binding sites of intrinsic dissociation constant 2.7×10^{-4}. It should be noted that in this interpretation the electrophoretic boundaries are considered to be due to the presence of two forms of the protein rather than to protein and complex, as was found to be the case in earlier studies (98, 100).

Other typical chemical modifications of proteins have been mentioned in previous sections (III and IV). In general, it is found that, outside of a change in mobility, chemical modification of proteins has little effect on their electrophoretic properties. A discussion of this is available (153).

B. Denaturation

Another type of transformation which occurs in proteins is that generally described as denaturation. This covers a broad spectrum of changes which vary in nature over a wide range but usually do not involve the breaking of protein covalent bonds. In general, the information revealed by Tiselius electrophoresis on denatured proteins is not very extensive and usually amounts to changes in mobility and the occasional formation of new moving boundaries. A review of this subject is available (154).

An interesting recent application of electrophoresis to the problem of denaturation can be found in the work of Tsiperovich and Loseva (155) who have studied the denaturation of ovalbumin in the presence of urea. From the results, shown in Fig. 21, the authors concluded that when ovalbumin is denatured in the presence of urea, the process is of an all-or-none type, since as denaturation progresses, the area under the native protein boundaries decreases gradually, while a new boundary attributed to denatured protein increases at the same time. From the data of Tsiperovich

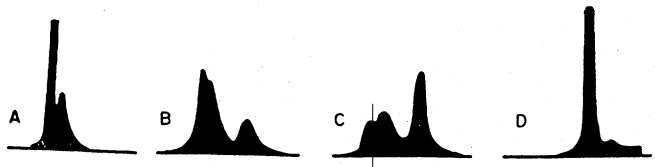


FIG. 21. Electrophoretic diagrams showing progress of ovalbumin denaturation in urea (159). Protein concentration: 2.33%; pH 7.2, 0.05 *M* phosphate buffer; urea concentration: 400 mg/ml; 180 minutes at 2.5 volts/cm. A, native ovalbumin; B, 30% denatured ovalbumin; C, 47.9% denatured ovalbumin; D, 95–100% denatured ovalbumin.

and Loseva it would seem also that the A_1 component is denatured more rapidly than the A_2 component, since the relative area under its peak decreases at a faster rate.

Briggs and Hull (156) have studied the kinetics of denaturation of β-lactoglobulin by Tiselius electrophoresis in a pH 6.9 phosphate buffer of 0.1 ionic strength. By following the change in component areas with time, they were able to conclude that at pH 7 two distinct processes occur in the denaturation of that protein. The first process, which is initiated at temperatures above 65°C., is accompanied by a fourfold increase in particle weight, but little change in electrophoretic properties. The second process, which can take place only after the first one has occurred, proceeds at lower temperatures and is accompanied by a marked increase in electrophoretic mobility and a large increase in particle weight.

Another important related application of electrophoresis is the study of gross composition changes in biological systems on exposure to various conditions. In some cases, rather profound changes in electrophoretic composition may occur even on exposure to mild conditions. In general, permanent changes in proteins under a variety of conditions have been observed by electrophoresis in a large number of systems. A few typical cases might be cited as examples. Longsworth (9) have found that ovalbumin undergoes a transformation on exposure to acid pH's with the formation of a new electrophoretic component. The denaturation of serum proteins on exposure to pH's below 4.0 has been studied electrophoretically by Perlmann and Kaufman (157) with the finding that a new component with a mobility intermediate between those of α_2- and β-globulins is formed. A single peak results from the denaturation of serum proteins with ultraviolet irradiation (158). This has been attributed to the unwinding of the coiled molecules. Tsiperovich and Loseva (159) have reported on the formation of a "stabilized form," with a higher electrophoretic mobility, of ovalbumin upon treatment with urea at pH 7.0. These are far from exhaust-

ing the vast literature which exists on similar electrophoretic transformations, but a complete coverage of the field is far beyond the scope of this chapter.

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